ACTINOMYCETES AND FUNGI ASSOCIATED WITH MARINE INVERTEBRATES: A POTENTIAL SOURCE OF BIOACTIVE COMPOUNDS

A Thesis
submitted in partial fulfilment
of the requirements for the degree
of
Doctor of Philosophy in Microbiology
at the
University of Canterbury
by

Nor Ainy Mahyudin

University of Canterbury
Christchurch, New Zealand

January 2008

Acknowledgements

I wish to thank my supervisors, Associate Professor Anthony Cole, Professor Murray Munro and Professor John Blunt for their help and guidance throughout the practical and written work of this thesis.

My thanks go to all the technical staff of the School of Biological Sciences at the University of Canterbury. Special thanks to Craig Galilee and Nic Cummings for their expertise in microbiology and to Neil Andrews for his assistance with Scanning Electron Microscopy. Thanks also to Matt Walters for his great assistance with photography and image scanning. Finally my thanks go to Graeme Young for his help and support for IT services and Dave Tattle for New Zealand marine sample collection.

I would especially like to thank Gill Ellis for biological assays and Dr. Marie Squire, Robert Stainthorpe and Bruce Clark for mass spectrometry analysis.

Many thanks to the Department of Fisheries Malaysia and the Ministry of Natural Resources and Environment Malaysia for their support in the work conducted in Malaysia, in particular Wan Norhana Noordin and Zaidnuddin Illias from Fisheries Research Institute, Pulau Pinang.

Current and former members of the Marine Chemistry Group have been invaluable for their help and guidance, particularly Annabel, Sun Lin, Maya, Gerhard, Sonia and Dilip. Many thanks to my friends in the School of Biological Sciences especially Shiva, Sunita and Termizi for their support.

I appreciate the financial support provided by the Public Service Department Malaysia and a Scholarship by the Government of Malaysia.

Finally my very special thanks to my very good friends Hajar and her husband Kamaruddin for their friendship, encouragement, moral and emotional support and great company.

Abstract

Actinomycetes and fungi were successfully isolated from both New Zealand and Malaysian marine invertebrates and classified as facultatively marine based on their ability to grow on both sea water and non-sea water media. Most of the extracts obtained from selected isolates were cytotoxic. A clear preference of the actinomycetes for solid-state fermentation was observed, however, for fungi no significant preference was seen. Three isolates of *Streptomyces* spp., four *Penicillium* spp. and two *Paecilomyces* spp. whose extracts showed good cytotoxicity were selected for further investigation.

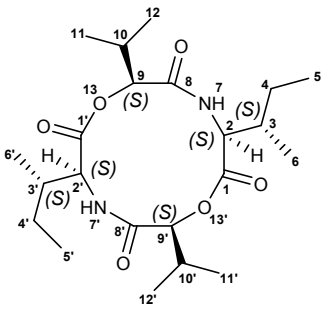
A small-scale extract obtained from a solid culture of *Streptomyces* sp. (LA3L2) showed good cytotoxicity and a new cytotoxic metabolite was isolated from a large-scale extract of *Streptomyces* sp. (LA3L2). This metabolite was characterized as *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate (**5.15**) and is only the third compound reported to contain the *S*-methyl benzothioate group. Two known compounds, montagnetol (**5.16**) and erythrin (**5.18**), were isolated from a further large-scale cultivation of *Streptomyces* sp. (LA3L2) and is the first reported actinomycete to produce these lichen-related compounds. In addition, two known inactive metabolites (bohemamine (**5.1**) and bohemamine B (**5.2**)) were identified from the small-scale extract. *Streptomyces* sp. (LA3L2) was also investigated for the effect of temperature and salinity on growth and cytotoxicity and shown to produce bohemamine only at 20 - 28ºC and 4% sea salt concentration on solid media. This isolate gave a low yield of active metabolite under all conditions.

Small-scale extracts of two other *Streptomyces* spp. yielded three known cytotoxic metabolites. These were thiazostatin B (**7.14**) from *Streptomyces* sp. (LA5L4) and chromomycin A2 (**7.1**), chromomycin A3 (**7.2**) and chromomycin 02-3D (**7.3**) from *Streptomyces* sp. (LA3L1).

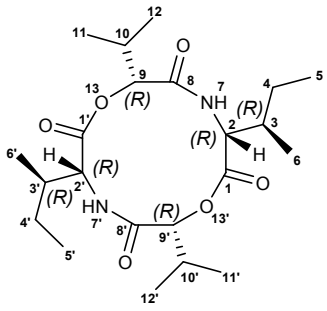
All four *Penicillium* spp. produced known metabolites. *Penicillium* sp. (LY1L5) yielded two known metabolites, cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**). α-Cyclopiazonic acid (**7.5**) and three other known metabolites (roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**)) were isolated from *Penicillum* sp. (KK3T23). *Penicillium* sp. (KK3T8) produced brefeldin A (**7.10**), while mycophenolic acid (**7.12**) and brevianamide A (**7.11**) were produced by *Penicillium* sp. (KK4T14b).

The effect of salinity on growth and cytotoxicity was investigated for the two *Penicillium* isolates producing the cytotoxic metabolite, α-cyclopiazonic acid (**7.5**). Saline conditions were not required for growth but metabolite production differed between the two isolates with respect to salinity. Isolate LY1L5 required saline conditions for α-cyclopiazonic production whereas isolate KK3T23 produced the metabolite under non-saline conditions and in concentrations of sea salt up to 6%.

Three known compounds, indole-3-carboxylic acid (**7.15**), indole-3-carboxylate (**7.17**) and 5-carboxymellein (**7.16**) were identified from *Paecilomyces* sp. (PR5L9). Investigation of a small-scale extract obtained from a solid culture of another *Paecilomyces* sp. (PR10T2) resulted in the isolation and characterization of a unique structure of a symmetrical cyclic depsipeptide, *epi*-angolide (NAM 6-1). NAM 6-1 was considered as a new compound based on four homoisomeric configurations (**A1, A2, A3 and A4**).



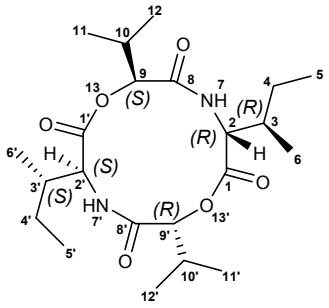
(A1)



(A2)

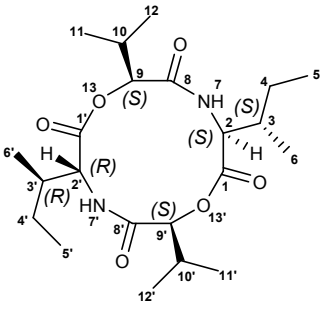
A1: (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl)

A2: (cyclo-D-α-hydroxyisovaleryl-D-isoleucyl-D-α-hydroxyisovaleryl-D-isoleucyl)



(A3)

(cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-D-α-hydroxyisovaleryl-D-isoleucyl)



(A4)

(cyclo-L-α-hydroxyisovaleryl-D-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl)

The value of dereplication procedures with respect to the rapid identification of metabolites and enhancement of in-house metabolite libraries is discussed. Structural elucidation of nine known metabolites (**7.1, 7.2, 7.3, 7.5, 7.6, 7.7, 7.8, 7.10** and **7.11**) was greatly aided by the in-house dereplication techniques using LC-MS-UV and AntiMarin database.

A significant advantage was gained by the use of the CapNMR which enabled NMR characterization of very small quantities of metabolites (<20 μg). Approximately <5 μg of materials were required to perform 1D proton NMR experiments for the dereplication of seven known compounds; bohemamine (**5.1**), bohemamine B (**5.2**), thiazostatin B (**7.14**), indole-3-carboxylate (**7.17**) and 5-carboxymellein (**7.16**).

Approximately 20 μg of materials were needed to acquire 1D and 2D (HSQC, HMBC and NOE) NMR spectra for structural elucidation of the new metabolite, *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate (**5.15**). Some 8 μg of materials were sufficient to perform 1D and 2D (COSY, HSQC and HMBC) NMR experiments for complete structural characterization of two known metabolites, montagnetol (**5.16**) and erythrin (**5.18**). Approximately 10 μg of materials were needed to acquire 1D and 2D NMR (COSY, HSQC and HMBC) experiments for structural elucidation of the new compound, *epi*-angolide NAM 6-1 (**A1, A2, A3** and **A4**).

Rapid identification of known fungal metabolites enabled the in-house HPLC-UV/R_t library to be enhanced by eight metabolites (**7.5, 7.6, 7.7, 7.8, 7.10, 7.11, 7.17** and **7.16**). An HPLC-UV/R_t library for actinomycete metabolites was successfully established with the insertion of eight known metabolites (**5.1, 5.2, 5.16, 5.18, 7.1, 7.2, 7.3** and **7.14**).

Abbreviations

°C	degrees celsius
1D	one dimensional
2D	two dimensional
δ	chemical shift in ppm (in NMR)
λ_{max}	maximum wavelength in nm
µL	microlitre
µm	micrometer
µg	microgram
acetone-d_6	deuterated acetone
ACD/Labs	Advanced Chemistry Development, Inc.
ACN	acetonitrile
aff.	affinity to
ATCC	American Type Culture Collection
br	broad (in NMR)
C18	octadecyl phase (chromatography column packing)
CapNMR	capillary-probe NMR
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
COSY	correlation spectroscopy (in NMR)
d	doublet (in NMR)
d	days
Da	dalton
DAD	diode array detector
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer (in NMR)
DMDCS	dimethyldichlorosilane
DMSO-d_6	deuterated dimethyl sulfoxide

ε	extinction coefficient
e.g.	example given
EI	electron ionization
ELSD	evaporative light scattering detector
ESIMS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
F-actin	filamentous actin
g	gram
h	hours
HCT-116	human colon tumor cells
HDO	deuterated water
HIV-1	human immunodeficiency virus 1
HMBC	heteronuclear multiple bond correlation spectroscopy (in NMR)
HPLC	high performance liquid chromatography
HRESIMS	high resolution electrospray ionization mass spectrometry
HRLCMS	high resolution liquid chromatography mass spectrometry
HSQC	heteronuclear single quantum coherence (in NMR)
Hz	hertz
IC_{50}	concentration of sample required to inhibit the growth of P388 cell growth by 50%
IR	infrared
J	coupling constant in Hz (in NMR)
kg	kilogram
kV	kilovolt
LC	liquid chromatography
LCMS	liquid chromatography mass spectrometry
log	logarithmic
m	metre
m	multiplet (in NMR)
mA	milliampere

m/z	mass-to-charge ratio (in MS)
mg	milligram
mL	millilitre
mm	millimeter
mV	millivolt
MeOH	methanol
MHz	megahertz
min	minute
mμ	millimicron
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
nm	nanometre
NMR	nuclear magnetic resonance
No.	number
NOE	nuclear overhauser effect (in NMR)
OD	optical density
OSMAC	one strain-many compounds
pA	picoampere
P388 cells	murine leukaemia cells
Pet. Ether	petroleum ether
PDA	photodiode array
ppm	parts per million
PTFE	polytetrafluoroethylene
PYGA	peptone yeast glucose agar medium
PYGA-CS	peptone yeast glucose agar-chlortetracycline/streptomycin medium
PYGB	peptone yeast glucose broth medium
q	quartet (in NMR)
QTOF	quadrupole time-of-flight
RPMI-1640	Roswell Park Memorial Institute-1640 medium
R_t	retention time

s	singlet (in NMR)
SCA	starch casein agar medium
SCA-N	starch casein agar-nystatin medium
SCA-NG	starch casein agar-nystatin/gentamycin medium
SCA-NN	starch casein agar-nystatin/novobiocin medium
SCB	starch casein broth medium
Scuba	self contained underwater breathing apparatus
SEM	scanning electron microscope
sp.	species (singular)
spp.	species (plural)
syn.	synonym
t	triplet (in NMR)
T cells	thymus cells
TFA	trifluoroacetic acid
TIC	total ion current
TOFMS	time-of-flight mass spectrometry
UV	ultraviolet
V	volt

Table of contents

Chapter 3: Preliminary screening

Chapter 4: New Zealand *Penicillium* spp.

Part D: Effect of temperature and salinity on growth and cytotoxicity of *Streptomyces* sp. (LA3L2)

Chapter 6: A Malaysian *Paecilomyces* sp. (PR10T2)

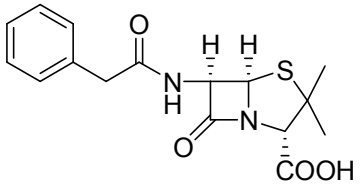
Chapter 7: Application of rapid techniques for chemical characterization of metabolites

Chapter 1

Introduction

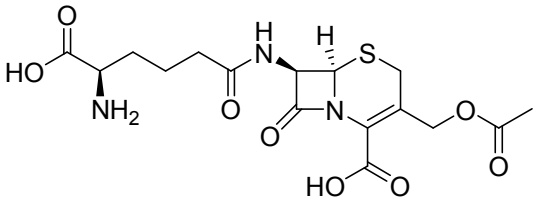
1.1 Marine natural products

Natural products, as the term implies, are chemical compounds originated from living organisms such as plants, animals and microorganisms. Traditionally, higher plants used to be the most prolific sources of drugs from nature and the use of medicinal plants is well documented throughout human history (Barton and Nakanishi, 1999). Marine folk medicine has been reported such as the seaweed remedies that were used by the Chinese and San Blas Indians in Panama, and the medicinal properties of some marine animals used by the Romans (Ruggieri, 1976). Although antibiotic properties of microorganisms were described in the late 1800s and early 1900s (Bugni and Ireland, 2004), it was later, when penicillin (**1.1**) was discovered in 1929 by Sir Alexander Fleming that microorganisms heralded the era of natural products.

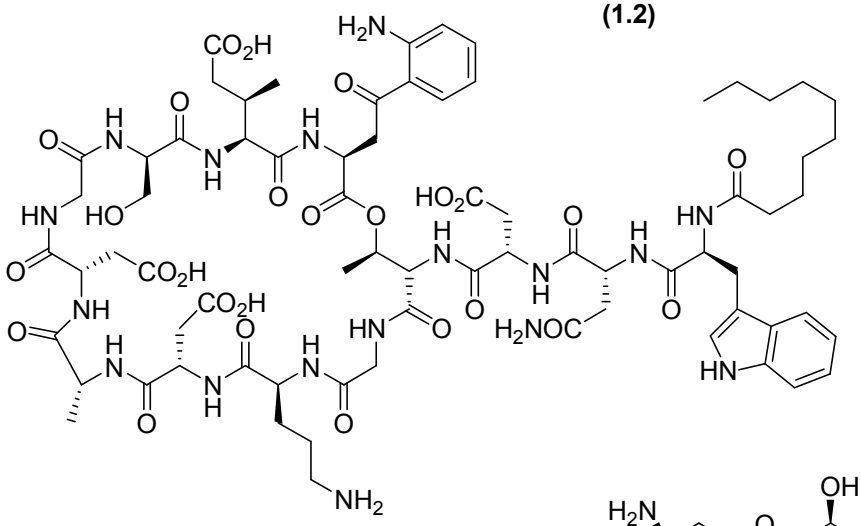


(1.1)

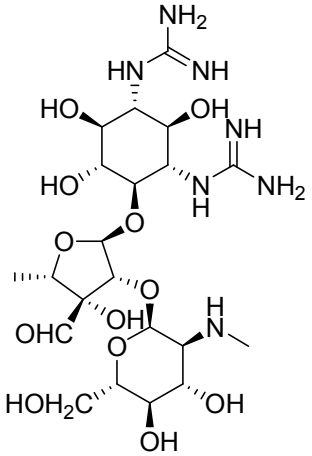
Since then, intensive studies of mainly soil-derived bacteria and fungi have shown that the microorganisms are a rich source of structurally unique pharmaceutically important bioactive substances (Fenical, 1993). Antibiotics, such as cephalosporin C (**1.2**), daptomycin (**1.3**), streptomycin (**1.4)**, vancomycin (**1.5**), the antifungals amphotericin B (**1.6**) and griseofulvin (**1.7**), the antivirals acyclovir (**1.8**) and doxorubicin (**1.9**) play a pivotal role in medicine.



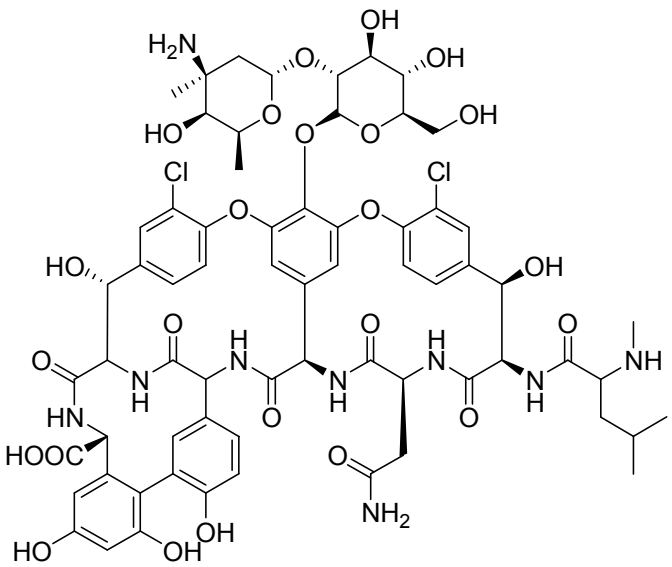
(1.2)



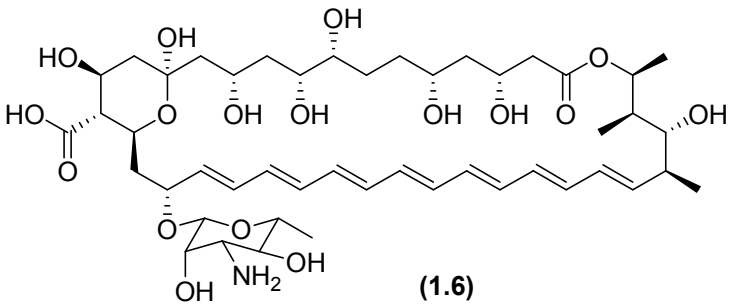
(1.3)



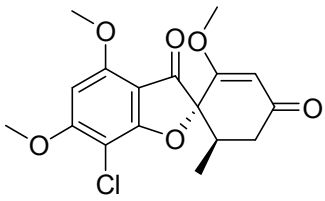
(1.4)



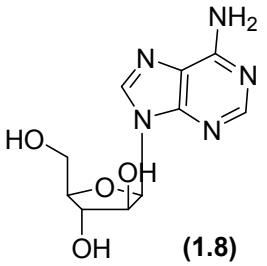
(1.5)



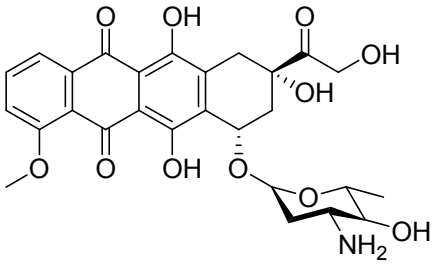
(1.6)



(1.7)

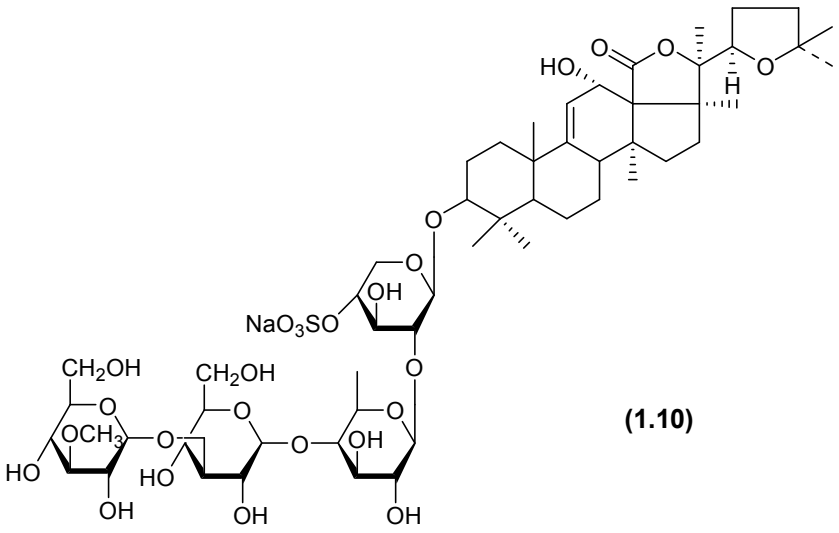


(1.8)



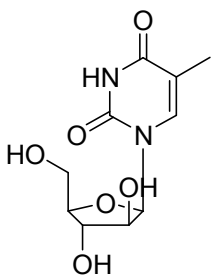
(1.9)

The rediscovery of high numbers of previously described metabolites has to some extent precluded the study of traditional terrestrial sources and has led researchers to explore unique habitats, such as the marine environment, for potentially new biosynthetic diversity. Scientists began to probe the oceans looking for sources of medicine in the middle of the 20th century. By the early 1950s, a toxin, later named holothurin (**1.10**) was reported from the Bahamian sea cucumber, *Actinopyga agassizi* (Atz, 1952; Nigrelli *et al*., 1967; Kitagawa *et al*., 1981). Although holothurin (**1.10**) was never commercialized, the search for drugs from marine sources continued.

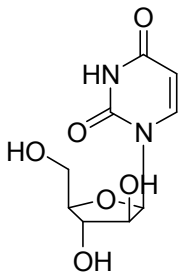


(1.10)

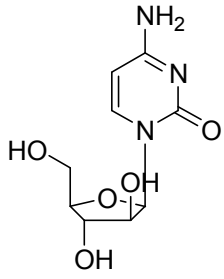
Since the discovery of the sponge-derived nucleotides, spongothymidine (**1.11**) and spongouridine (**1.12**) (Bergmann and Feeney, 1951), it became evident that the ocean is a likely source of pharmaceuticals with a number of marine natural compounds now being marketed such as cytarabine (**1.13**) (also known as Ara-C) mainly used in the treatment of lymphomas and also for acute myelogenous leukemia, or are under clinical trials for many different diseases (Haefner, 2003). Some are undergoing clinical trials for treatment of cancer, analgesia, allergy, and cognitive diseases (Newman and Cragg, 2004).



(1.11)



(1.12)



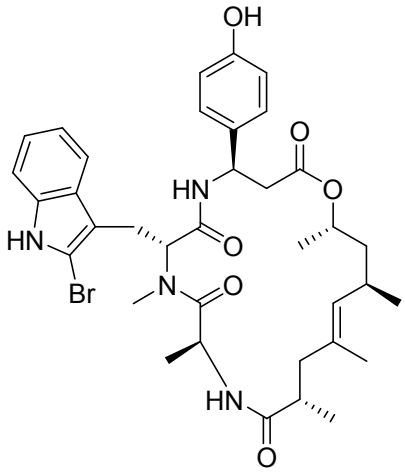
(1.13)

The potential of marine microorganisms as a source of antibiotics began after penicillin (**1.1**) was developed as a human antibiotic in the early 1940s. The earliest report indicating marine microorganisms as antibiotic producers occurred in 1945 when Giuseppe Brotzu described antibacterial activity of a fungus obtained from seawater samples (Abraham and Loder, 1972). The fungus identified as *Cephalosporium acremonium* (now named *Acremonium chrysogenum*) produced cephalosporin C (**1.2**). Studies reporting natural products from marine-derived microorganisms were few until the 1990s, however, a remarkable growth occurred through the 1990s (Faulkner, 2000a). Marine microorganisms continue to be a productive and successful focus for much marine natural product research (Faulkner, 2000b; Faulkner, 2001; Faulkner, 2002; Blunt *et al*., 2003; Blunt *et al*., 2004; Bugni and Ireland, 2004; Blunt *et al*., 2005; Blunt *et al*., 2006; Blunt *et al*., 2007).

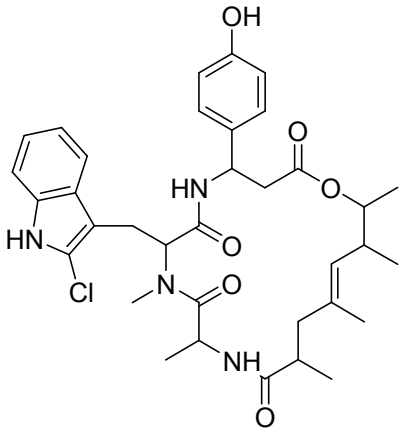
1.2 Marine microbe-invertebrate association

Marine microorganisms are not taxonomically defined, but are classified according to their habitat and their physiology. This ecological definition has gained general acceptance by taxonomist (Furuya, 1986) although pragmatically, marine microorganisms can also be defined as microbes that have been isolated from marine sources on marine media (Faulkner, 1999). In marine habitats these microbes may occur suspended, on living or inanimate surfaces of epibionts, or as symbionts. Marine organisms such as the invertebrates are known to be important reservoirs for microorganisms. Marine invertebrates are those animals that lack of notochord, a characteristic of vertebrates and these include sponges, jellyfish, corals, sea stars, brittle stars and sea fans. Most marine invertebrates harbour microorganisms within their tissues where they reside in the extra- and intra-cellular spaces (Wilkinson, 1992). These become classical substrates for the isolation of marine microorganisms.

The striking similarity of some microbial metabolites and natural products from marine invertebrates has aroused much speculation in the past. The possibility that some metabolites derived from marine animals might originate from symbiotic bacteria living within the host tissues rather than from the host animals themselves has, therefore, been discussed intensely for many years (Bewley and Faulkner, 1998; Moore, 1999; Proksch *et al*., 2002; Piel, 2004). An example is the cyclodepsipeptide jasplakinolide (**1.14**) (syn. jaspamide), isolated from marine sponges (*Jaspis* spp). It is used as a molecular tool in cell biology being a cell permeable F-actin probe (Bubb *et al*., 1994) and shows a close structural relationship to chondramide D (**1.15**) known from the myxobacterium *Chondromyces crocatus* (Jansen *et al*., 1996).

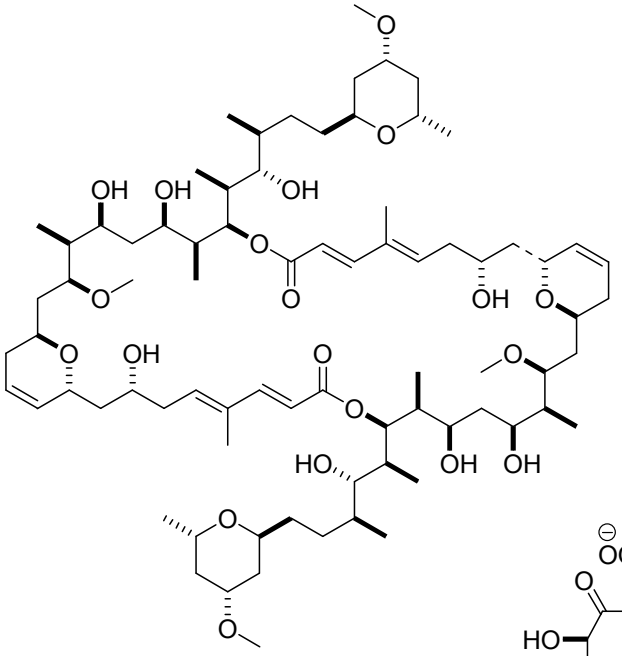


(1.14)

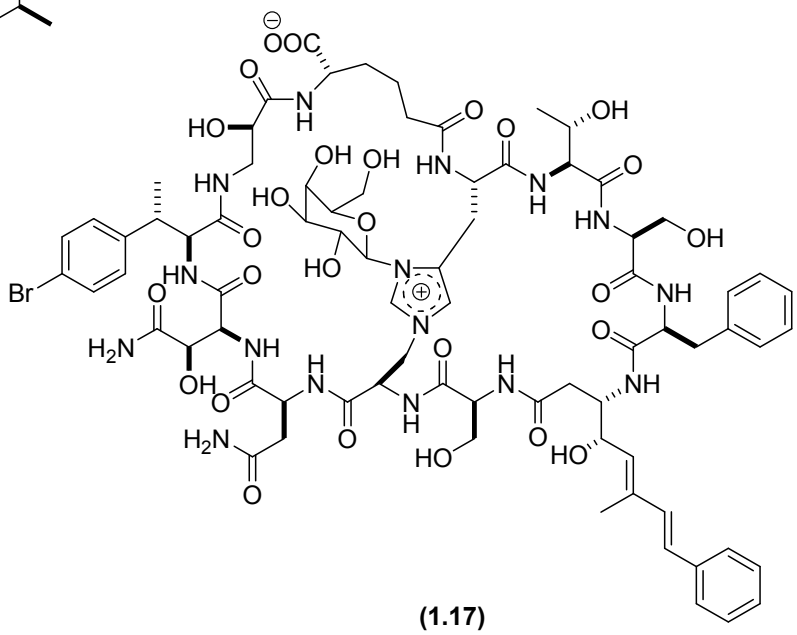


(1.15)

Further evidence of symbiotic producers was demonstrated in a study on the associations of the sponge *Theonella swinhoei* with bacteria (Bewley *et al*., 1996). The study showed that the cytotoxic polyketide swinholide A (**1.16**) was mainly present in unicellular bacteria, while the antifungal peptide theopalauamide (**1.17**) was detected in a filamentous bacterium.



(1.16)



(1.17)

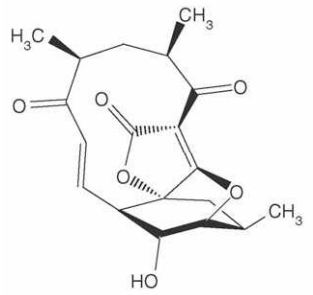
Marine microbes appeared to have adapted excellently to the marine environment, thus have gained special attention because of their diverse metabolic capabilities and their divergent physiologic lifestyles. Marine ecosystems contain several unique features that set them apart from other aquatic ecosystems, the main factor being the presence of dissolved compounds in seawater, particularly sodium chloride (NaCl). Salinity is expressed as the amount (in grams) of total dissolved salts present in 1 kg of water. Normal seawater with a salinity of 35 g/kg (or litre) of water is expressed as 35‰. Microorganisms, particularly fungi and yeasts are well known for their ability to grow in saline environments by adapting their osmoregulatory mechanisms that signal the production of osmolytes (e.g: polyols, amino acids) in conjunction with an increasing concentration of cytoplasmic ions (Harris, 1981; Bloomberg and Adler, 1992; Roberts, 2005). For marine microorganisms, their cellular adaptation to moderate and high salt content is a fundamental biological process needed for survival and growth. It is postulated that marine microorganisms have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds.

1.3 Marine actinomycetes

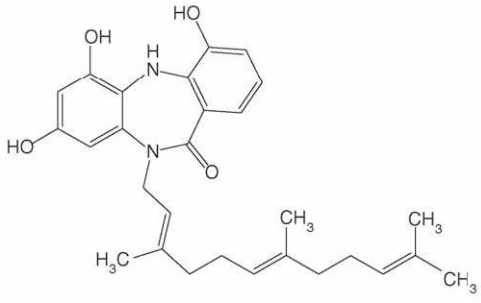
Actinomycetes has been isolated from different niches of the marine realm, such as mangroves and the deep sea (Weyland, 1969; Walker and Colwell, 1975; Barcina *et al*., 1987; Jensen *et al*., 1991; Rathnakala and Chandrika, 1993; Imada and Okami, 1995; Colquhoun *et al*., 1998; Stritzke *et al*., 2004) and from macroorganisms such as tunicates (Charan *et al*., 2004), and sponges (Lee *et al*., 1998; Lee *et al*., 2005). Earlier it was believed that marine actinomycetes were of terrestrial origin, however later it has become apparent that many are indigenous components of the marine world.

The definition of true marine actinomycetes is difficult but the ability of actinomycetes to grow in a marine environment has been demonstrated, notably in connection with their occurrence in salt-rich muds and sea areas close to the shore. Earlier studies showed that *Streptomyces* spp. can grow at 2 and 16% NaCl, while various species of *Streptomyces* and *Nocardia* grow well in the presence of 10% NaCl (Waksman, 1959). More recently, other studies have shown that various actinomycetes derived from marine sediments show good growth in the presence of NaCl (Jensen *et al*., 1991; Mincer *et al*., 2002; Kokare *et al*., 2004; Magarvey *et al*., 2004). Recently, the occurrence of an obligate *Micromonospora* sp. (Mincer *et al*., 2002) and *Salinospora* sp. (Mincer *et al*., 2002; Feling *et al*., 2003; Maldonado *et al*., 2005) has been reported.

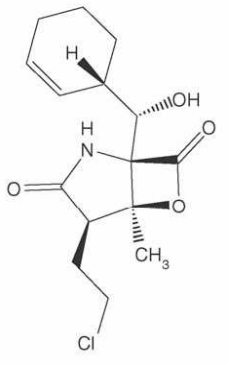
Among the microorganisms, actinomycetes were the most studied group because of their capacity to produce novel bioactive secondary metabolites. Actinomycetes are primarily saprophytic and are best known from soils where they contribute significantly to the turnover of complex biopolymers (Williams *et al*., 1984). Despite their importance in soil ecology, the role of actinomycetes as potential antibiotic producers became apparent in 1940, following the discovery of actinomycin (Waksman and Woodruff, 1940), and was fully realized by the 1980s, when actinomycetes accounted for almost 70% of the world's naturally occurring antibiotics (Okami and Hotta, 1988). The decline in the discovery of new compounds form common soil-derived actinomycetes in the past two decades led to the attempted cultivation of rare or novel actinomycete taxa (Bull *et al*., 2000; Mincer *et al*., 2002). Although the exploitation of marine actinomycetes as a source for novel secondary metabolites is in its infancy, the discovery rate of novel secondary metabolites from marine actinomycetes has recently surpassed that of their terrestrial counterparts, as evident by the isolation of many different diverse structures in the past few years (Fiedler *et al*., 2005; Jensen *et al*., 2005a). Some examples of particular interest are abyssomicin C (**1.18**), diazepinomicin (**1.19**) and salinosporamides (**1.20**).



(1.18)



(1.19)



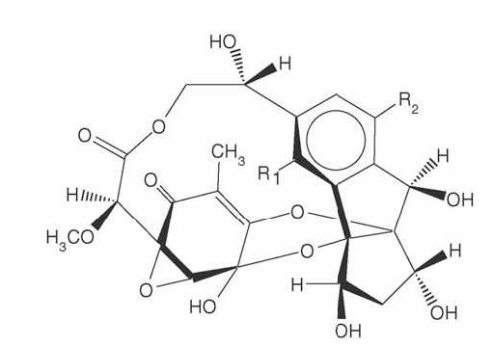
(1.20)

Abyssomicin C (**1.18**) is produced by a marine *Verrucosispora* strain (Riedlinger *et al*., 2004). This novel polycyclic polyketide antibiotic is a potent inhibitor of para-aminobenzoic acid biosynthesis and therefore, inhibits folic acid biosynthesis at an earlier stage than the well-known synthetic sulfa drugs (Bister *et al*., 2004; Lam, 2006). Abyssomicin C (**1.18**) shows potent activity against Gram-positive bacteria, including clinical isolates of multiple-resistant and vancomycin-resistant *Staphylococcus aureus* (Lam, 2006). Abyssomicin C (**1.18**) or its analog (Rath *et al*., 2005) has the potential to be developed as an antibacterial agent against drug-resistant pathogens (Lam, 2006).

Diazepinomicin (**1.19**) (ECO-4601) is a metabolite produced by a *Micromonospora* strain (Charan *et al*., 2004) that possesses antibacterial, anti-inflammatory and antitumor activity. This unique farnesylated dibenzodiazepinone has a broad spectrum of *in vitro* cytotoxicity and has demonstrated *in vivo* activity against glioma, breast and prostate cancer in mouse models. The preclinical development of ECO-4601 as an anticancer agent has been completed by Ecopia BioSciences Inc. Ecopia filed Clinical Trial Application (equivalent to Investigational New Drug application in the USA) for ECO-4601 in Canada on 3 January 2006 (Lam, 2006).

A novel β-lactone-γ-lactam, salinosporamide A (**1.20**) (NPI-0052) has been isolated from a fermentation broth of a new obligate marine actinomycete, *Salinispora tropica* (Feling *et al*., 2003; Maldonado *et al*., 2005). This compound (**1.20**) is an orally active proteasome inhibitor that induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug Bortezomib (Chauhan *et al*., 2005). NPI-0052 is being developed by Nereus Pharmaceuticals, Inc. and represents the first clinical candidate for the treatment of cancer produced by saline fermentation of an obligate marine actinomycete (Lam, 2006).

Strains of *S. tropica* also produced two unique macrolides, besides the production of NPI-0052 (**1.20**). These macrolides, sporolides A (**1.21a**) and B (**1.21b**) (Buchanan *et al*., 2005) appear to be synthesized from two different polyketides containing a large number (23 out of 24 carbon skeleton) of oxidized carbons. Even though sporolides have not demonstrated antimicrobial and anticancer activity, these unusual structures of sporolides demonstrate the potential of marine actinomycetes for the production of novel secondary metabolites (Lam, 2006).



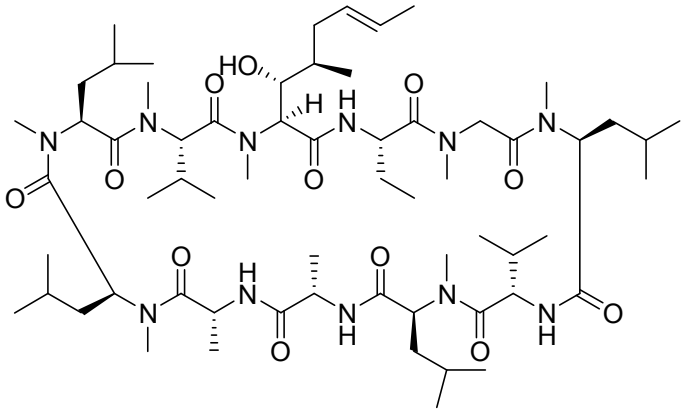
(1.21a): R_1 = Cl, R_2 = H
(1.21b): R_1 = H, R_2 = Cl

The potential for the isolation of novel secondary metabolites from marine actinomycetes relies on the ability to isolate novel actinomycetes from marine environments. Some progress in the isolation strategies have been made recently. These techniques using enrichment, new selection methods and media (Magarvey *et al*., 2004; Jensen *et al*., 2005b; Mincer *et al*., 2005) have led to the isolation of novel actinomycetes derived from sediment. Further development work in improving isolation strategies in the recovery of marine actinomycetes from various sources is of paramount importance for ensuring success in this area.

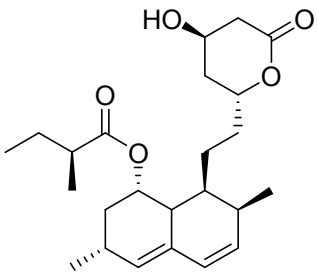
1.4 Marine fungi

In the past, marine fungi have often been defined based on their ability to grow at certain seawater concentrations. As the definition cannot be strictly based on physiological aspects (Kohlmeyer, 1974; Hughes, 1975), a broad ecological definition was used namely; *obligate marine fungi* are those that grow and sporulate exclusively in a marine or estuarine habitat; *facultative marine fungi* are those from freshwater or terrestrial environment able to grow (and possibly also to sporulate) in the marine environment (Kohlmeyer, 1974).

After the discoveries of penicillin (**1.1**) and cephalosporin C (**1.2**), a further milestone in the history of medicinal fungal products was the discovery of cyclosporin A (**1.22**) produced by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Dreyfuss *et al*., 1976). The antifungal agent griseofulvin (**1.7**) was isolated from *Penicillium griseofulvum* (Rehm, 1980) and the cholesterol biosynthesis inhibitor, lovastatin (**1.23**), isolated from *Aspergillus terreus* (Alberts *et al*., 1980) are two further metabolites of great pharmacological importance from fungi.

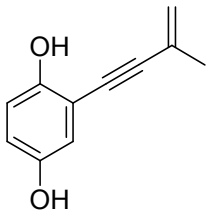


(1.22)



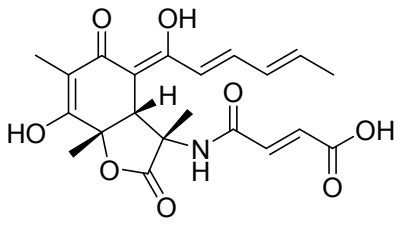
(1.23)

Although siccayne (**1.24**) (Fenical, 1997) was most likely the first antibiotic isolated from an obligate marine fungus (Bugni and Ireland, 2004), the compound had been previously isolated from a terrestrial species (Kupka *et al*., 1981). Overall, research on marine-derived fungi has led to the discovery of some 272 new natural products until 2002 (Bugni and Ireland, 2004), and another 240 new structures from 2002 until 2004 (Ebel, 2006), thus, providing evidence that marine-derived fungi have a potential to be a rich source of pharmaceutical leads.



(1.24)

Bugni and Ireland (2004) showed that fungi from marine invertebrates (sponges, corals and tunicates) showed the largest contribution to the total number of compounds described in the literature (40%) with the fungi from marine plants, driftwood or sediments accounting for 37%. The review by Ebel (2006) shows the majority of novel fungal secondary metabolites originate from inorganic matter (soil, sediments, sandy habitats and artificial substrates), while marine invertebrate-derived fungi contributed less than the marine plants (algae, sea grasses, mangrove plants and woody habitats). It noted that even if many of the marine-derived fungi are already known from terrestrial habitats, and have been chemically well studied, novel secondary metabolites continue to be detected. An example is the marine-derived *Penicillium chrysogenum* that was isolated from the sponge, *Ircinia fasciculate*. This isolate produced the sorbicillin-derived alkaloid sorbicillacton A (**1.25**) (Bringmann *et al*., 2003) that showed selective cyctostatic activity against lymphoblasts and was able to protect human T cells against HIV-1. Preclinical evaluation of this compound has recently been initiated (Proksch *et al*., 2006). Some of the examples of fungal metabolites isolated from sponges, corals and tunicates are listed in Table 1.1. Many of these fungi have novel carbon skeletons thus providing further evidence of the potential of marine-derived fungi.



(1.25)

Table 1.1: Some examples of new metabolites from fungi derived from marine invertebrates (1994 – 2007).

Fungi	Marine invertebrates	Metabolites	Activities	References
unidentified	sponge (*Stylotella* sp.)	nectriapyrones A (**1.26**) and B (**1.27**)	ns	Abrell *et al*., 1994
unidentified	sponge (*Jaspis* aff. Johnstoni)	chloriolins A (**1.28**), B (**1.29**) and C (**1.30**)	ns	Cheng *et al*., 1994
unidentified	sponge (*Spirastrella vagabunda*)	14,15-secocurvularin (**1.31**)	ns	Abrell *et al*., 1996
Gymnascella dankaliensis	sponge (*Halichondria japonica*)	gymnastatins A (**1.32**), B (**1.33**) and C (**1.34**)	cytotoxic	Numata *et al*., 1997; Amagata *et al*., 1998
Coniothyrium sp.	sponge (*Ectyplasia perox*)	(3*S*)-(3',5'-dihydroxyphenyl) butan-2-one (**1.35**) 2-(1'(*E*)-propenyl)-octa-4(*E*),6(*Z*)-diene-1,2-diol (**1.36**)	antimicrobial inactive	Holler *et al*., 1999
Microsphaeropsis sp.	sponge (*Myxilla incrustans*)	microsphaeropsisin (**1.37**)	antimicrobial	Holler *et al*., 1999
Aspergillus niger	tunicate (*Aplidium* sp.)	yanuthones A (**1.38**), B (**1.39**), C (**1.40**), D (**1.41**) and E (**1.42**) 1-hydroxy-yanuthone A (**1.43**) and C (**1.44**) 22-deacetyl-yanuthone A (**1.45**)	antimicrobial	Bugni *et al*., 2000
Drechslera hawaiiensis	sponge (*Callyspongia aerizusa*)	spiciferol A (**1.46**) monocyclic spiciferone derivative (**1.47**)	ns	Edrada *et al*., 2000
unidentified	sponge	*iso*-cladospolide B (**1.48**) and *seco*-patulolide C (**1.49**), pandangolides 1 (**1.50**) and 2 (**1.51**)	inactive	Smith *et al*., 2000
Hortaea werneckii	sponge (*Aplysina aerophoba*)	hortein (**1.52**)	inactive	Brauers *et al*., 2001
Cladosporium herbarum	sponge (*Callyspongia aerizusa*)	pandangolides 3 (**1.53**) and 4 (**1.54**)	inactive	Jadulco *et al*., 2001
Penicillium cf. *montanense*	sponge (*Xestospongia exigua*)	xestodecalactone B (**1.56**) xestodecalactones A (**1.55**) and C (**1.57**)	antifungal inactive	Edrada *et al*., 2002
Cladosporium herbarum	sponge (*Aplysina aerophoba*)	herbarins A (**1.58**) and B (**1.59**)	inactive	Jadulco *et al*., 2002
Curvularia lunata	sponge (*Niphates olemda*)	lunatin (**1.60**)	antibacterial	Jadulco *et al*., 2002
Penicillium citrinum	sponge	isocyclocitrinol A (**1.61**) and 22-acetylisocyclocitrinol A (**1.62**)	weak antibacterial	Amagata *et al*., 2003
Aspergillus versicolor	sponge (*Xestospongia exigua*)	aspergillitine (**1.63**) aspergiones A (**1.64**), B (**1.65**), C (**1.66**), D (**1.67**), E (**1.68**) and F (**1.69**)	moderate antibacterial inactive	Lin *et al*., 2003
Penicillium brevicompactum	sponge (*Petrosia ficiformis*)	petrosifungins A (**1.70**) and B (**1.71**)	ns	Bringmann *et al*., 2004
Aspergillus niger	sponge (*Axinella damicornis*)	3,3′-bicoumarin bicoumanigrin (**1.72**) aspernigrin A (**1.73**) aspernigrin B (**1.74**) pyranonigrins A (**1.75**), B (**1.76**), C (**1.77**) and D (**1.78**)	moderate cytotoxicity inactive neuroprotective inactive	Hiort *et al*., 2004
Penicillium sp.	sponge (*Axinella verrucosa*)	communesins C (**1.79**) and D (**1.80**)	moderate antiproliferative	Jadulco *et al*., 2004
Phomospis asparagi	sponge (*Rhaphidophlus juniperina*)	chaetoglobosins-510 (**1.81**) and -540 (**1.82**) chaetoglobosin-542 (**1.83**)	antimicrofilament cytotoxic	Christian *et al*., 2005
Gymnascella dankaliensis	sponge (*Halichondria japonica*)	gymnastatins F (**1.84**) and G (**1.85**) gymnastatin H (**1.86**)	cytotoxic inactive	Amagata *et al*., 2006a
unidentified	sponge (*Ianthella* sp.)	guangomides A (**1.87**) and B (**1.88**)	weak antibacterial	Amagata *et al*., 2006b
Acremonium sp.	sponge *(Teichaxinella* sp.)	RHM1 (**1.89**) and RHM2 (**1.90**)	weak cytotoxicity	Boot *et al*., 2006
Penicillium rugulosum	sponge	prugosenes A1 (**1.91**), A2 (**1.92**), A3 (**1.93**), B1 (**1.94**), B2 (**1.95**), C1 (**1.96**), and C2 (**1.97**)	inactive	Lang *et al*., 2007
Penicillium aurantiogriseum	sponge (*Mycale plumose*)	aurantiomide A (**1.98**) aurantiomides B (**1.99**) and C (**1.100**)	inactive moderate cytotoxicity	Xin *et al*., 2007

ns – not stated

1.5 Screening approaches

Biological and chemical screening of natural products is one of the earliest steps in drug discovery. In the 1950s and 1960s, most bioassays targeting diseases other than bacteria and fungal infections involved whole animal models of disease and these assays were not suitable for the study of complex mixtures such as natural products (Borris and Gould, 1999). Chemical screening was rather primitive, with chromatography meant gravity-fed open column or thin layer plates, spectroscopy meant infrared (IR) and ultraviolet (UV) and with mass spectrometry (MS) and nuclear magnetic resonance (NMR) was only beginning to come into routine use. Grams of pure compounds were required for complete identification and initial biological evaluation.

Technology has changed since then with the development of various biological and chemical techniques, thus substantially compressing the time cycle while the sample requirements have been drastically reduced. The mode of action of current assays is fast, selective, quantitative and readily amenable to automation. High Performance Liquid Chromatography (HPLC) has been routinely employed in separation techniques and greatly facilitates assay guided isolation of active metabolites. While IR and UV spectra are still in use, modern spectroscopic method such as high resolution MS, multidimensional NMR and capillary-probe NMR (CapNMR) greatly simplified the identification of the metabolites. The discovery of novel metabolites in marine microorganisms has become more difficult due to the enormous number of known compounds already described in the literature. It is most important therefore to rapidly identify and exclude the known compounds at the earliest possible stage. This process is called dereplication, where properties of mixtures or pure metabolites are compared with literature data. Some widely used dereplication techniques are based on reverse-phase liquid chromatography (LC) combined with diode array detection (Frisvad and Thrane, 1987; Yoshimura *et al.*, 1994) or with MS (Landreau *et al*., 2002) or with UV/MS (Nielsen and Smedsgaard, 2003) or with MS/CapNMR (Hu *et al*., 2005). These techniques are conducted efficiently in combination with the rapidly growing chemical databases of known compounds (e.g: AntiMarin, AntiBase, SciFinder and others).

1.6 Scope of present study

The main goals of the present investigation are the isolation of marine-derived actinomycetes and fungi, the application of techniques for chemical characterization of their metabolites and the effect of salinity on their growth and cytotoxicity.

1.6.1 Isolation of marine actinomycetes and fungi

Actinomycetes and fungi were isolated from New Zealand and Malaysian marine invertebrates. Tissue or liquid portions of these invertebrates were used in obtaining the isolates. Selected isolates were investigated for secondary metabolites production.

1.6.2 Assessment of culture extracts for biological activity

Small scale cultivation and extraction enabled biological activity testing. These tests mainly consisted of cytotoxic assay against P388 murine leukaemia cell lines (P388 cells) and antimicrobial assay against the Gram-positive bacterium *Bacillus subtilis*, the Gram-negative bacterium *Pseudomonas aeruginosa* and the fungus *Candida albicans*. Cytotoxic and antimicrobial tests were undertaken. Cytotoxicity was assayed against the P388 cells and antimicrobial activity against the bacteria and fungus.

1.6.3 Chemical characterization of bioactive compounds

Rapid techniques were used to dereplicate and characterize new metabolites. Small-scale or large-scale cultivation, extraction, and separation using chromatographic methods, mainly HPLC, was employed, in order to isolate pure metabolites. Structural elucidation was performed using mainly one dimensional (1D) or two dimensional (2D) CapNMR techniques.

1.6.4 Physiological studies on growth and production of metabolites

The effect of salinity on growth and metabolite production of two *Penicillium* isolates was undertaken. The effect of salinity and temperature on growth and metabolite production of *Streptomyces* sp. was also carried out.

Chapter 2

Experimental

2.1 Isolation and cultivation media

The antifungal agent, nystatin was incorporated in all actinomycetes isolation media. Combination of nystatin/gentamycin or nystatin/novobiocin was also used. Combination of the antibiotic solutions (chlortetracycline and streptomycin) was used in all fungal isolation media. Both isolation and cultivation media were adjusted to pH 7.0 (±0.2) prior to sterilization. Mineral salt constituents were prepared fresh before use. Antibiotic/antifungal solutions were stored frozen at -20ºC.

2.1.1 Media for actinomycetes

2.1.1.1 Starch casein agar medium (SCA)

5.0 g soluble starch (BDH), 0.2 g casein (Sigma), 1.0 g KNO_3 (BDH), 1.0 g NaCl (BDH), 1.0 g K_2HPO_4 (Sigma), 0.5 mL $MgSO_4$ solution, 0.5 mL $CaCO_3$ solution, 0.5 mL $FeSO_4$ solution, 10.0 g agar (Oxoid) and 20.0 g sea salt (Sigma) in 500 mL distilled water.

2.1.1.2 Starch casein agar-nystatin medium (SCA-N)

5.0 g soluble starch (BDH), 0.2 g casein (Sigma), 1.0 g KNO_3 (BDH), 1.0 g NaCl (BDH), 1.0 g K_2HPO_4 (Sigma), 0.5 mL $MgSO_4$ solution, 0.5 mL $CaCO_3$ solution, 0.5 mL $FeSO_4$ solution, 10.0 g agar (Oxoid) and 12 mL nystatin solution (Sigma) in 500 mL natural seawater (100%).

2.1.1.3 Starch casein agar-nystatin/gentamycin medium (SCA-NG)

5.0 g soluble starch (BDH), 0.2 g casein (Sigma), 1.0 g KNO_3 (BDH), 1.0 g NaCl (BDH), 1.0 g K_2HPO_4 (Sigma), 0.5 mL $MgSO_4$ solution, 0.5 mL $CaCO_3$ solution, 0.5 mL $FeSO_4$ solution, 10.0 g agar (Oxoid), 12 mL nystatin solution (Sigma) and 500 μL gentamycin (Sigma) solution in 500 mL natural seawater (100%).

2.1.1.4 Starch casein agar-nystatin/novobiocin medium (SCA-NN)

5.0 g soluble starch (BDH), 0.2 g casein (Sigma), 1.0 g KNO_3 (BDH), 1.0 g NaCl (BDH), 1.0 g K_2HPO_4 (Sigma), 0.5 mL $MgSO_4$ solution, 0.5 mL $CaCO_3$ solution, 0.5 mL $FeSO_4$ solution, 10.0 g agar (Oxoid), 12 mL nystatin solution and 1.0 mL novobiocin solution in 500 mL natural seawater (100%).

2.1.1.5 Starch casein broth medium (SCB)

5.0 g soluble starch (BDH), 0.2 g casein (Sigma), 1.0 g KNO_3 (BDH), 1.0 g NaCl (BDH), 1.0 g K_2HPO_4 (Sigma), 0.5 mL $MgSO_4$ solution, 0.5 mL $CaCO_3$ solution, 0.5 mL $FeSO_4$ solution and 20.0 g sea salt (Sigma) in 500 mL distilled water.

2.1.2 Media for fungi

2.1.2.1 Peptone yeast glucose agar medium (PYGA)

0.5 g peptone (Becton Dickinson), 0.5 g yeast extract (Oxoid), 1.0 g glucose (BDH), 10.0 g agar (Oxoid) and 20.0 g sea salt (Sigma) in 500 mL distilled water.

2.1.2.2 Peptone yeast glucose agar-chlortetracycline/streptomycin medium (PYGA-CS)

0.5 g peptone (Becton Dickinson), 0.5 g yeast extract (Oxoid), 1.0 g glucose (BDH), 10.0 g agar (Oxoid) and 5.0 mL antibacterial solution in 500 mL natural seawater (100%).

2.1.2.3 Peptone yeast glucose broth medium (PYGB)

0.5 g peptone (Becton Dickinson), 0.5 g yeast extract (Oxoid), 1.0 g glucose (BDH) and 20.0 g sea salt (Sigma) in 500 mL distilled water.

2.2 Sampling of marine invertebrates

2.2.1 New Zealand samples

The samples from Kaikoura and in Lyttleton Harbour, New Zealand were collected and identified by Mr. Dave Tattle of the School of Biological Sciences, University of Canterbury. Details of the sampling location are shown in Figure 2.1. Nine samples (four from Kaikoura and five from Lyttleton Harbour) were collected and listed in Table 2.1. Samples were kept at -20ºC for 24 - 72 h prior to preparation.

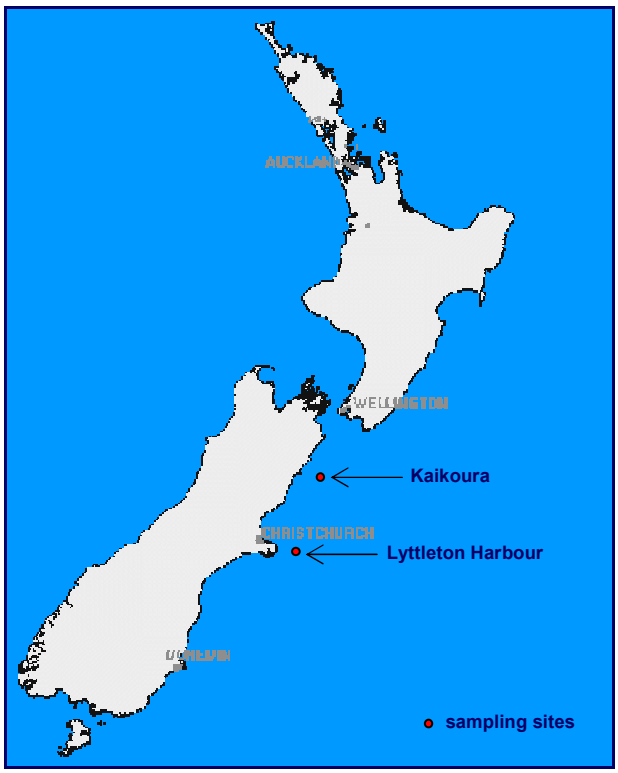


Figure 2.1: Sampling of New Zealand marine invertebrates showing the sampling sites at Kaikoura and Lyttleton Harbour.

Table 2.1: List of marine invertebrates collected from New Zealand waters.

Sampling sites	Marine invertebrates
Kaikoura	Sponge K2
	Sponge K3
	Golf ball sponge K4
	Colonial tunicate K8
Lyttleton Harbour	Tunicate L1
	Sponge L2
	Tunicate L3
	Golf ball sponge L4
	Sponge L5

2.2.2 Malaysian samples

Permission for collecting samples in Pulau Redang and Pulau Payar Marine Parks Malaysia was granted by the Director-General of Fisheries Malaysia (Fisheries Act 1985). The samples from these two marine protected areas were collected and identified by Mr. Zainuddin Illias of Fisheries Research Institute and his Scuba divers team from the Ministry of Natural Resources and Environment Malaysia. The samples were collected at a depth between 10 – 50 m at two sampling sites (see Figure 2.2). A total of 17 samples were collected (14 from Pulau Redang Marine Park and three from Pulau Payar Marine Park) and listed in Table 2.2. The samples (14 marine inverterbrates) from Pulau Redang Marine Park were processed immediately at the Pulau Redang Wet Lab (Figure 2.3). The three samples from Pulau Payar Marine Park were kept at 0ºC for 24 h prior to preparation at Fisheries Research Institute, Penang.

Figure 2.2: Sampling of Malaysian marine invertebrates showing the sampling sites at Pulau Redang and Pulau Payar Marine Parks.

Table 2.2: List of marine invertebrates collected from Malaysian waters.

Sampling sites	Marine invertebrates
Pulau Redang Marine Park	Tunicate PR1 (*Didemnum* sp.)
	Soft coral tree PR2 (*Dendronephthya* sp.)
	Soft coral tree PR3 (*Dendronephthya* sp.)
	Nudibranch PR4 (*Phyllidia* sp.)
	Sea fan PR5
	Sea whip PR7 (*Juncella* sp.)
	Yellow sponge PR8
	Soft coral PR9
	Sea fan PR10
	Soft sponge PR11
	Barrel sponge PR12 (*Xestospongia* sp.)
	Brittle star PR13
	Sponge PR14
	Staghorn coral PR17
Pulau Payar Marine Park	Soft coral tree PP1 (*Dendronephthya* sp.)
	Soft coral tree PP2 (*Dendronephthya* sp.)
	Sea anemone PP4

Figure 2.3: Immediate preparation of marine invertebrate samples at the Pulau Redang Wet Lab.

2.3 Preparation of marine invertebrate samples

Liquid and/or tissue portions of the marine invertebrates were used for the isolation of actinomycetes and fungi. In the case of some delicate samples, such as the tunicates, only the liquid portion was used. For samples such as hard corals (homogenization process was not applicable), a swabbing method was used. All marine invertebrate samples (fresh or frozen) were rinsed three times with sterile seawater before use.

For the preparation of the liquid portion, samples were aseptically cut into smaller pieces (±10 mm^3). The pieces were then placed in a mortar and homogenized with 10 mL sterile seawater with a pestle. An aliquot of the liquid portions (150 – 200 μL) was spread on each of 60 plates containing isolation media for actinomycetes (SCA-N, SCA-NG and SCA-NN) or fungi (PYGA-CS).

For the preparation of the tissue portion, samples were aseptically cut into smaller pieces (±3 mm^3) with stainless steel scissors. The tissue pieces (five – seven pieces) were aseptically placed onto each of 60 plates containing isolation media for actinomycetes (SCA-N, SCA-NG and SCA-NN) or fungi (PYGA-CS).

The inoculated plates (20 each) were then incubated at 20, 28 and 37°C. For the immediately prepared samples (Pulau Redang Marine Park samples), the inoculated plates were maintained at 20ºC during the sample preparation and below 10ºC upon return from the island to the Fisheries Research Institute laboratory in Penang. Isolation plates were examined daily and developing colonies were transferred to fresh isolation plates until pure cultures obtained (see Figure 2.4).

2.4 Maintenance of pure cultures

For long-term maintenance, pure isolates of actinomycetes and fungi were maintained in 10% glycerol at -80°C. Cultures under regular examination were transferred monthly on a fresh medium (SCA – actinomycetes or PYGA – fungi) and kept at 20°C. The plates were wrapped with parafilm or plastic wrap to reduce drying.

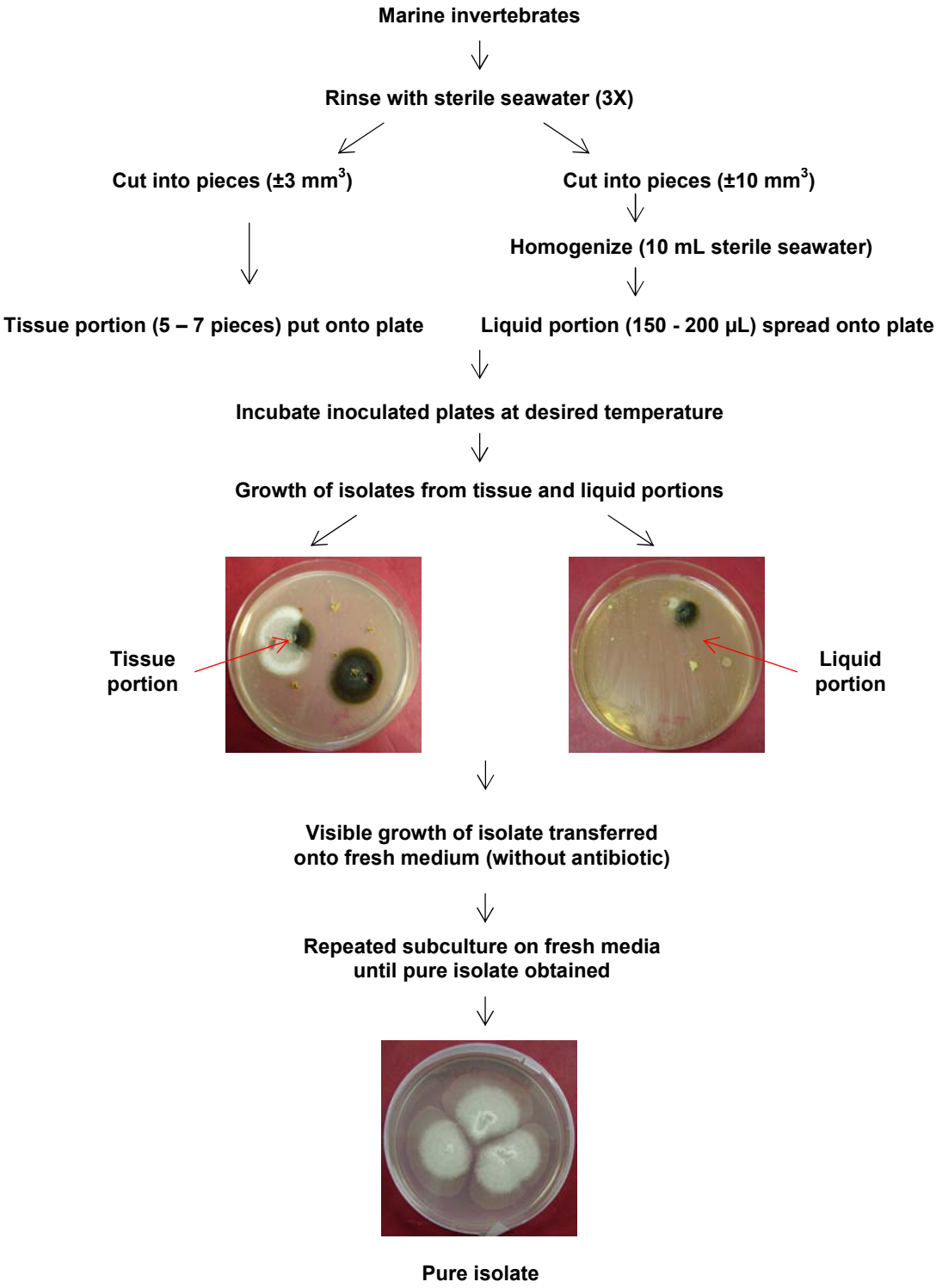


Figure 2.4: General technique to isolate actinomycetes or fungi from tissue/liquid portions of marine invertebrates (adopted from Namikoshi *et al.*, 2002).

2.5 Identification of isolates

Isolates were examined macroscopically and microscopically. The morphology of isolates was examined under a dissection microscope. Microscopic details such as hyphal reproductive structures and spores were examined using a compound microscope with magnification up to 400X. For slide preparation for examination under the compound microscope, a small piece of surface tissue from the colony was scraped and placed into a drop of 70% ethanol (EtOH) on a microscope slide. Once the EtOH had evaporated, a drop of lactophenol cotton blue was applied to the specimen. A coverslip was then gently placed over the drop of stain and pressed lightly. For long-term storage of the slide, the coverslip was ringed with nail polish, repeated several times.

Higher resolution images of these structures were obtained using a scanning electron microscope (SEM) with the aid of Mr. Neil Andrews, School of Biological Sciences, University of Canterbury. A mounted stub with double sided sticky tape was plunged into the culture area bearing spores. The specimen was then coated with a thin coating of Gold Palladium using a Polaron E5000 coater with the following conditions: 1.2 kV at 20 mA for 2 min. Micrographs were taken by a Lieca S440 at 10 kV and 80 pA with working distance approximately 10 mm.

2.6 Extraction

2.6.1 Agar media

Cultures of actinomycetes and fungi (both agar and mycelium) on agar plates (85 mm diameter) were cut into pieces and homogenized with EtOAc (25 mL/plate) using an Ultra-turrax (Janke & Kunkle) for 5 min or until the sample was sufficiently macerated. The ground agar was left sitting in the EtOAc overnight. The EtOAc was then pipetted off from the macerated agar using a micropipette. This process was repeated three times. The filtered EtOAc (from three extractions) were combined and taken to dryness under N_2 (for large scale extraction, the extracts were dried on a rotary evaporator). The concentrated extract was transferred to a pre-weighed vial, dried under N_2 and the dry weight determined. All dried extracts were dissolved in HPLC grade methanol (MeOH) at a concentration of 1 mg/mL prior to submission for bioassay or HPLC screening.

2.6.2 Broth media

Broth cultures of actinomycetes and fungi (10 mL) were homogenized with 10 mL EtOAc using an Ultra-turrax (Janke & Kunkle) for 5 min or until the samples were sufficiently macerated. The ground broth culture was left sitting in EtOAc overnight. The EtOAc was then filtered off from the macerated broth using a micropipette. This process was repeated three times. The filtered EtOAc (from three extractions) was combined and taken to dryness under N_2. The concentrated extract was transferred to a pre-weighed vial, dried under N_2 and the dry weight determined. All dried extracts were dissolved in a HPLC grade MeOH at a concentration of 1 mg/mL prior to submission to the bioassay or HPLC screening.

2.7 Bioassays

2.7.1 P388 Assay

All crude extracts (1 mg/mL) were initially screened for cytotoxicity against murine leukaemia cell lines P388 (P388 cells) (ATCC CCL 46, P388D1). An aliquot of 5 µL of each crude extracts was pipetted into 90 individual wells of a 96-well microtitre plate. This assay comprised of a serial dilution of the sample of interest followed by incubation for 72 h with P388 cells. Cell viability is determined colorimetrically by the addition of the yellow dye, MTT tetrazolium. Unhealthy or dead cells cannot metabolize this dye, leaving a yellow colour, whereas healthy cells reduce this dye to MTT formazan resulting in an intense purple colour. The concentration of sample required to reduce cell growth by 50% when compared to controls, is expressed as an IC_{50} in ng/mL (Perry *et al*., 1999). Samples that inhibited 50% of the growth of P388 cells were considered as potentially active and were subjected to a further assay to determine the concentrations required to inhibit the growth of P388 cells by 50% (IC_{50}).

2.7.2 Antimicrobial Assays

Crude extracts were tested against the Gram-positive bacterium *Bacillus subtilis*, the Gram-negative bacterium *Pseudomonas aeruginosa* and the fungus *Candida albicans*. Bacterial and fungal suspensions were prepared in Roswell Park Memorial Institute-1640 medium (RPMI-1640). The density of cells was read at 610 nm and adjusted to an optical density (OD) of 0.04 for *B. subtilis*, and 0.05 for *P. aeruginosa* and *C. albicans*. Aliquots of 200 µL of bacterial or fungal suspension were incubated with each crude extract for 24 h at 30ºC. The cell viability was determined colorimetrically by the addition of the blue dye, resazurin. Resazurin is a growth indicator and the presence of bacteria or fungi is indicated by oxygen emission which reacts with resazurin, generating a pink colour.

The UV absorption of each sample was read at 600 nm and compared against the UV absorption of the media control. Results are given as a percentage of cell viability (Lang *et al.*, 2006). For the antimicrobial assay against bacteria, an aliquot of 10 µL of each crude extract (1 mg/mL) was pipetted into 90 individual wells of a 96-well microtitre plate. For the assay against fungi, 20 µL was pipetted into 90 individual wells of a 96-well microtitre plate.

2.8 Capillary-probe nuclear magnetic resonance (CapNMR)

^{1}H, correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation spectroscopy (HMBC) and nuclear overhauser effect (NOE) experiments were all recorded on a Varian INOVA 500 spectrometer at 23°C, operating at 500 MHz. The INOVA was equipped with a 500 MHz Protasis capillary NMR probe (Figure 2.5) for spectra reported in **Chapters 5, 6** and **7**. Chemical shifts are expressed in parts per million (ppm) on the δscale, and were referenced to the appropriate solvent peaks: $CDCl_3$ referenced to C**H**Cl_3 at δ_H 7.25 (^{1}H); CD_3OD referenced to C**H**D_2OD at δ_H 3.31 (^{1}H); DMSO-d_6 referenced to CD_3(C**H**D_2)SO at δ_H 2.50 (^{1}H). Coupling constants (J) were expressed in Hz.

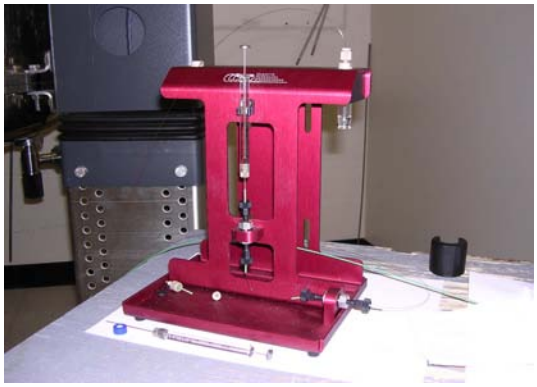


Figure 2.5: Protasis capillary NMR probe.

The pure or partially pure compound obtained from the method described in **Section 2.9.3** was dissolved in 6 µL of deuterated MeOH and injected into an inlet capillary via a syringe. This was then followed by injection of 11 µL of deuterated MeOH to position the sample in the probe. All samples were retrieved into HPLC vials or inserts that were silated as described in **Section 2.12**. The formula for calculating the amount of material analyzed by the CapNMR is shown below.

$$A\ (\mu g) = \frac{\text{molecular weight of A}}{\sum \text{protons of A}} \times \frac{\sum \text{protons (solvent – A)}}{\sum \text{protons (solvent)}} \times 0.35$$

2.9 High pressure liquid chromatography (HPLC)

Analytical HPLC was carried out on a Dionex liquid chromatograph equipped with a UVD 340U diode array detector (DAD), and connected to an Alltech evaporative light scattering detector (ELSD) 800. For reverse-phase HPLC a Phenomenex Luna C18 (10 x 250 mm, 5 µm) column was used. The standard gradient used was: 2 min of 10% ACN/H_2O; a linear gradient to 75% ACN/H_2O for 12 min; isocratic at 75% for another 10 min; a linear gradient for 2 min to 100% ACN/H_20 followed by isocratic at 100% ACN for 4 min then returned to 10% ACN/H_20 in 2 min and re-equilibriated for 8 min with a flow rate of 1 mL/min and at 40°C.

2.9.1 HPLC screening of metabolites

Bioactive samples were filtered through a 0.45 μm polytetrafluoroethylene (PTFE) membrane filters immediately prior to the HPLC screening. An aliquot of the samples (25 μg) was analyzed by reverse-phase analytical HPLC using the standard gradient solvent system given in **Section 2.9**. For HPLC screening, the milli-Q H_2O was acidified with 0.05% trifluoroacetic acid (TFA). The HPLC screening for significant peaks provides a basis for selection of extracts for further investigation. The presence of significant amount of metabolites was indicated by both the UV and ELSD traces (>10% of both the base peaks in the HPLC chromatogram).

2.9.2 HPLC microtitre plate bioassay

The HPLC MTT plate assay was conducted on selected extracts to determine the peak(s) responsible for the activity. An aliquot of the crude extract (250 μg) was analyzed by reverse-phase analytical HPLC using the standard gradient solvent system given in **Section 2.9**. For HPLC Microtitre Plate Bioassay, the milli-Q H_2O was acidified with 0.05% TFA. The eluent was collected into 96 well polystyrene microtitre plates (master plate). 'Daughter plates' were made by taking an amount of 50 or 5 μL from the master plate to a new plate. These plates containing the eluents were then used for bioassay.

2.9.3 HPLC microtitre plate for CapNMR

Sample for CapNMR analysis (500 – 750 μg) was separated by reverse-phase analytical HPLC using the standard gradient solvent system given in **Section 2.9**. The fractions were collected into 88 individual wells of a 96-well microtitre polypropylene plate and were dried using a centrifugal evaporator. The amount of sample collected from single or combined wells was determined by the ELSD traces of the chromatogram. A sample was considered worthwhile for further analysis if the traces showed at least 500% of the ELSD base peak. The pure or partially pure compound obtained from individual or combined wells of the plate was then subjected to CapNMR analysis.

2.10 Liquid chromatography mass spectrometry (LCMS)

High Resolution Liquid Chromatography Mass Spectra (HRLCMS) were recorded on a Waters 2790 HPLC system equipped with a Waters 996 photodiode array (PDA) detector coupled to a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150°C and a source temperature of 80°C. The carrier solvent was 50:50 ACN/H_2O at 20 μL/min (for direct inject mode). Typically, 10 μL of a 10 μg/mL solution was injected. Leucine enkephalin was used as the internal standard. The measurements were performed by Dr. Marie Squire, Mr. Robert Stainthorpe and Mr. Bruce Clark (Department of Chemistry, University of Canterbury).

2.11 Electrospray ionization mass spectrometry (ESIMS)

High Resolution Electrospray Ionization Mass Spectra (HRESIMS) were recorded on a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150°C and a source temperature of 80°C. The carrier solvent was 50:50 ACN/H_2O at 20 μL/min (for direct inject mode). Typically, 10 μL of a 10 μg/mL solution was injected. Leucine enkephalin was used as the internal standard. Electrospray ionization mass spectra (ESIMS) reported in **Chapters 5, 6** and **7** were recorded in positive and/or negative modes on a Micromass (Manchester, UK) quadrupole time of flight (QTOF-2) mass spectrometer. The measurements were performed by Dr. Marie Squire, Mr. Robert Stainthorpe and Mr. Bruce Clark (Department of Chemistry, University of Canterbury).

2.12 Silanization of glass surfaces for CapNMR

All items were immersed into dimethyldichlorosilane (DMDCS) solution (5% DMDCS in dichloromethane (DCM)) for 15 min. After pouring off the deactivating solution, the items were immediately rinsed with DCM. The items were then immediately covered with MeOH for 15 – 30 min. This step was performed immediately in order to minimize exposure to room moisture. The MeOH was drained off and the items dried in a 100ºC oven until completely dry. The DMDCS solution was prepared fresh before use.

2.13 Solvents

All solvents used in the chromatography technique were HPLC grade. Analytical grade solvents were used for other methods.

Chapter 3

Preliminary screening

3.1 Introduction

The culturable microorganisms associated with marine invertebrates from New Zealand and Malaysian waters were explored for bioactivity and chemical interest. Within the Marine Chemistry Group at University of Canterbury, New Zealand, an integrated approach between research groups in microbiology and natural product chemistry is taken for marine natural products discovery. This chapter presents the groundwork that was conducted towards the search for biologically active metabolites from extracts of actinomycetes and fungi derived from marine invertebrates.

3.2 Actinomycetes and fungi isolated from marine invertebrates

Actinomycetes and fungi were isolated from New Zealand marine invertebrates collected off Kaikoura and in Lyttleton Harbour. Malaysian fungal strains were isolated from marine invertebrates collected in Pulau Redang Marine Park and Pulau Payar Marine Park. A total of 390 isolates were obtained from New Zealand (33 actinomycetes and 179 fungi) and Malaysian samples (178 fungi).

The isolates were initially identified based on the morphology and cultural characteristic and due to a great number of isolates, only the genus was determined and in some cases, the isolates remained unidentified. Details of the procedures for sampling, isolation, cultivation and identification of isolates were discussed in **Chapter Two** (*Experimental*).

3.2.1 New Zealand marine-derived actinomycetes

Thirty-three isolates were obtained from seven marine invertebrates (Table 3.1). The majority of the actinomycetes were isolated from tissue portions of the four sponges: K2, K3, K4 and L2. Actinomycetes were not isolated from the remaining two invertebrates, namely tunicate L1 and golf ball sponge L4.

Table 3.1: Actinomycetes isolated from New Zealand marine invertebrates.

Sampling sites	Marine invertebrates	Portion used	No. of isolates	Isolation media	Total
Kaikoura	Sponge K2	Tissue	5	SCA-N	5
		Liquid	0		
	Sponge K3	Tissue	4	SCA-N	4
		Liquid	0		
	Golf ball sponge K4	Tissue	4	SCA-N	4
		Liquid	0		
	Colonial tunicate K8	Liquid	4	SCA-NG	4
Lyttleton Harbour	Tunicate L1	Liquid	0		0
	Sponge L2	Tissue	5	SCA-NG	5
		Liquid	0		
	Tunicate L3	Tissue	0		2
		Liquid	2	SCA-N	
	Golf ball sponge L4	Tissue	0		0
		Liquid	0		
	Sponge L5	Tissue	4	SCA-NG	9
		Liquid	5	SCA-NG	
		Total			**33**

3.2.2 New Zealand marine-derived fungi

A total of 179 isolates were obtained from nine New Zealand marine invertebrates studied (see Table 3.2). It was noted that their abundance in tissue and liquid portions varied significantly in a number of cases.

Table 3.2: Fungi isolated from New Zealand marine invertebrates.

Sampling sites	Marine invertebrates	Portion used	No. of isolates	Total
Kaikoura	Sponge K2	Tissue	13	17
		Liquid	4	
	Sponge K3	Tissue	15	28
		Liquid	13	
	Golf ball sponge K4	Tissue	14	23
		Liquid	9	
	Colonial tunicate K8	Liquid	20	20
Lyttleton Harbour	Tunicate L1	Liquid	11	11
	Sponge L2	Tissue	4	13
		Liquid	9	
	Tunicate L3	Tissue	12	25
		Liquid	13	
	Golf ball sponge L4	Tissue	4	14
		Liquid	10	
	Sponge L5	Tissue	15	28
		Liquid	13	
Total				**179**

3.2.3 Malaysian marine-derived fungi

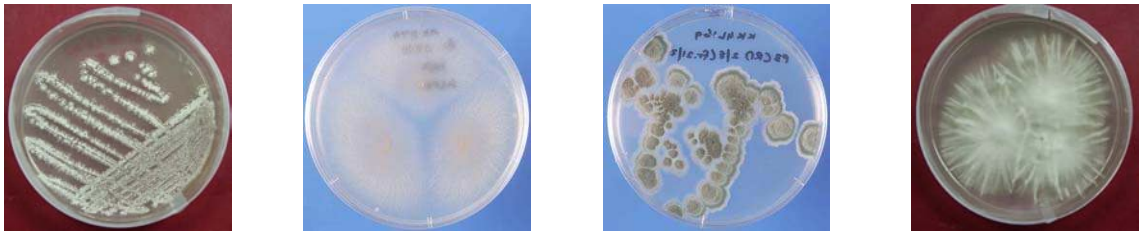
The isolation of fungi from Malaysian marine invertebrates resulted in a large number of isolates but because of time limitation only 178 fungi were obtained in pure culture from a total of 17 marine invertebrates (see Table 3.3).

Table 3.3: Fungi isolated from Malaysian marine invertebrates.

Sampling sites	Marine invertebrates	Portion used	No. of isolates	Total
Pulau Redang Marine Park	Tunicate PR1 (*Didemnum* sp.)	Tissue	3	7
		Liquid	4	
	Soft coral tree PR2 (*Dendronephthya* sp.)	Tissue	10	17
		Liquid	7	
	Soft coral tree PR3 (*Dendronephthya* sp.)	Tissue	18	25
		Liquid	7	
	Nudibranch PR4 (*Phyllidia* sp.)	Tissue	8	8
	Sea fan PR5	Tissue	8	15
		Liquid	7	
	Sea whip PR7 (*Juncella* sp.)	Tissue	3	3
	Yellow sponge PR8	Liquid	8	8
	Soft coral PR9	Tissue	4	5
		Liquid	1	
	Sea fan PR10	Tissue	8	20
		Liquid	12	
	Soft sponge PR11	Tissue	12	18
		Liquid	6	
	Barrel sponge PR12 (*Xestospongia* sp.)	Tissue	1	3
		Liquid	2	
	Brittle star PR13	Tissue	4	4
	Sponge PR14	Tissue	17	21
		Liquid	4	
	Staghorn coral PR17	Liquid	4	4
Pulau Payar Marine Park	Soft coral tree PP1 (*Dendronephthya* sp.)	Tissue	7	7
	Soft coral tree PP2 (*Dendronephthya* sp.)	Liquid	5	5
	Sea anemone PP4	Tissue	2	8
		Liquid	6	
Total				**178**

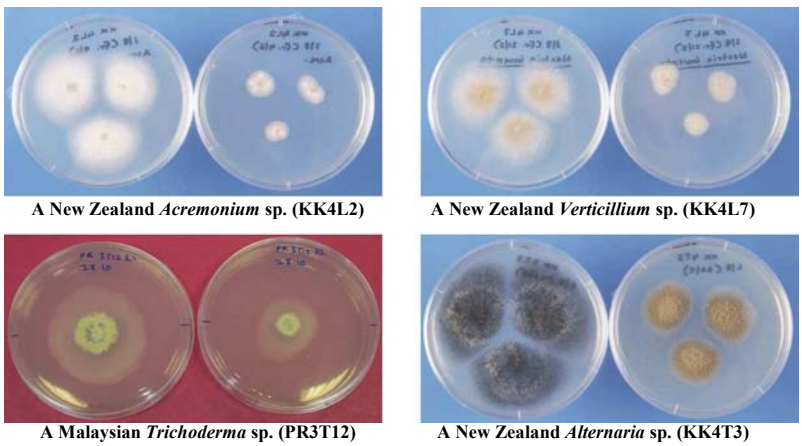
3.3 Selection of isolates for bioactivity screening

From the 390 isolates obtained from New Zealand and Malaysian marine invertebrates only 161 were further evaluated for the production of biologically active metabolites. The selection was based on the taxonomy of the isolates, unusual morphology and the diversity of reported secondary metabolite chemistry of related taxa. Some of the isolates were also randomly selected from productive genera such as *Streptomyces*, *Acremonium, Penicillium* and *Xylaria*. Fungal isolates that showed better growth on sea water media were also selected. Some examples of the selected isolates are shown in Figures 3.1 (a) and (b).



A New Zealand *Streptomyces* sp. (KA4T1) | **A New Zealand *Acremonium* sp. (KK2T4)** | **A New Zealand *Penicillium* sp. (KK4T14b)** | **A Malaysian *Xylaria* sp. (PR3L8)**

(a): Examples of isolates selected for bioactivity screening based on productive genera.



A New Zealand *Acremonium* sp. (KK4L2) | **A New Zealand *Verticillium* sp. (KK4L7)**

A Malaysian *Trichoderma* sp. (PR3T12) | **A New Zealand *Alternaria* sp. (KK4T3)**

(b): Examples of isolates selected for bioactivity screening based on growth on sea water and non-sea water media. Left plates: sea water medium. Right plates: non-sea water medium.

Figures 3.1: Examples of isolates selected for bioactivity screening.

Of the 161 isolates selected, 13 isolates were New Zealand actinomycetes, 91 New Zealand fungi and 57 Malaysian fungi. Each of the New Zealand actinomycetes was cultured on SCA and SCB media; the New Zealand fungal isolates were cultured on PYGA and PYGB media. For the Malaysian fungal isolates, 17 and 41 of the isolates were cultured on PYGA and PYGB media, respectively. Resultant cultures were extracted with EtOAc to yield a total of 266 extracts (see Table 3.4). All 266 extracts were "quick screened" against P388 cells, *B. subtilis*, *P. aeruginosa* and *C. albicans*. Extracts that inhibited >50% of the growth of P388 cells were further assayed to determine the concentrations required to inhibit the growth of the cells by 50% (IC_{50}). Details of the cultivation, extraction and bioactivity screening procedures are given in **Chapter Two** (*Experimental*). The results are discussed below.

Table 3.4: Number of extracts produced from selected isolates cultured on agar and/or broth media.

Origins of isolate	No. of isolates selected	Agar media	Broth media	Total
Sponge K2	4	4	4	8
Sponge K3	2	2	2	4
Golf ball sponge K4	1	1	1	2
Sponge L2	2	2	2	4
Tunicate L3	2	2	2	4
Sponge L5	2	2	2	4
Sub-total (New Zealand actinomycetes)	***13***	***13***	***13***	***26***
Sponge K2	10	10	10	20
Sponge K3	16	16	16	32
Sponge K4	14	14	14	28
Tunicate K8	4	4	4	8
Tunicate L1	6	6	6	12
Sponge L2	12	12	12	24
Tunicate L3	12	12	12	24
Sponge L4	12	12	12	24
Sponge L5	5	5	5	10
Sub-total (New Zealand fungi)	***91***	***91***	***91***	***182***
Tunicate PR1 (*Didemnum* sp.)	4	1	3	4
Soft coral tree PR2 (*Dendronephthya* sp.)	4	1	3	4
Soft coral tree PR3 (*Dendronephthya* sp.)	9	3	6	9
Nudibranch PR4 (*Phyllidia* sp.)	7	1	6	7
Sea fan PR5	5	1	4	5
Sea whip PR7 (*Juncella* sp.)	2	2	0	2
Sponge PR8	2	1	1	2
Soft coral PR9	2	1	1	2
Sea fan PR10	4	2	2	4
Soft sponge PR11	1	0	1	1
Barrel sponge PR12 (*Xestospongia* sp.)	1	0	1	1
Brittle star PR13	3	2	2	4
Sponge PR14	1	1	0	1
Staghorn coral PR17	2	0	2	2
Soft coral tree PP1 (*Dendronephthya* sp.)	4	0	4	4
Soft coral tree PP2 (*Dendronephthya* sp.)	2	0	2	2
Sea anemone PP4	4	1	3	4
Sub-total (Malaysian fungi)	***57***	***17***	***41***	***58***
Total	**161**	**121**	**145**	**266**

3.4 Bioactivity of actinomycetes and fungi

3.4.1 New Zealand actinomycetes

The quick screen assay of the New Zealand actinomycetes showed that 25 of the 26 extracts assayed were potentially active (growth inhibition >50%). Of the 25 extracts, 23 inhibited only the growth of P388 cells and two were active against both P388 cells and *B. subtilis*. None of the extracts showed activity against *P. aeruginosa* or *C. albicans*. All 25 extracts were further assayed for IC_{50} against P388 cells and 9 of 25 extracts showed IC_{50} values of <12,500 ng/mL. Two extracts (F5956 and F5934) showed a high level of cytotoxicity (IC_{50}<97.5 ng/mL) and good cytotoxicity was shown by F5958 (IC_{50}784 ng/mL). The results of bioactivity are shown in Table 3.5.

Table 3.5: Bioactivity of New Zealand actinomycete extracts.

Isolates	Media	Extracts	A	B	C
KA2T2 (unidentified)	SCB	F5921	84.0	na	na
	SCA	F5943	88.9	na	na
KA2T5 (unidentified)	SCB	F5922	85.0	na	na
	SCA	F5944	na	na	na
KA2T7 (unidentified)	SCB	F5923	64.6	na	na
	SCA	F5945	65.4	na	na
KA2T9 (unidentified)	SCB	F5924	88.0	na	na
	SCA	F5946	101.4	na	9,563
KA3T1 (*Streptomyces* sp.)	SCB	F5925	83.4	na	na
	SCA	F5947	100.6	na	na
KA3T3 (*Streptomyces* sp.)	SCB	F5926	100.0	na	na
	SCA	F5948	98.0	na	6,303
KA4T1 (*Streptomyces* sp.)	SCB	F5928	85.4	na	5,938
	SCA	F5950	81.6	na	na
LA2T4 (unidentified)	SCB	F5932	64.2	na	na
	SCA	F5954	78.9	na	5,938
LA2T5 (unidentified)	SCB	F5933	97.0	na	na
	SCA	F5955	93.8	na	na
LA3L1 (*Streptomyces* sp.)	SCB	F5934	93.7	99.1	<97.5
	SCA	F5956	94.5	101.0	<97.5
LA3L2 (*Streptomyces* sp.)	SCB	F5936	79.8	na	na
	SCA	F5958	81.5	na	784
LA5T2 (unidentified)	SCB	F5937	80.2	na	na
	SCA	F5959	86.9	na	2,094
LA5L4 (*Streptomyces* sp.)	SCB	F5940	93.5	na	na
	SCA	F5962	90.2	na	1,339

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

3.4.2 New Zealand fungi

The quick screen assay of the New Zealand fungi showed that 94 of the 182 extracts assayed were potentially active (growth inhibition >50%). Of the 94 extracts, 89 were cytotoxic only against P388 cells, three were active against both P388 cells and *B. subtilis*, one was active against both P388 cells and *C. albicans* and the remaining extract was active only against *B. subtilis*. None of the extracts showed activity against *P. aeruginosa*. Of the total 93 extracts that were active against P388 cells in the quick screen assay, 32 showed IC_{50} values of <12,500 ng/mL. Good cytotoxicity (IC_{50}<97.5 ng/mL) was indicated in extract F5342 in particular, although a number of other extracts also showed promising results. The results of bioactivity are shown in Table 3.6.

Table 3.6: Bioactivity of New Zealand fungal extracts.

Isolates	Media	Extracts	A	B	C	D
KK 2T4 (*Acremonium* sp.)	PYGB	F5963	na	na	na	na
	PYGA	F5907	70.7	na	na	na
KK2T9 (*Acremonium* sp.)	PYGB	F5364	na	na	na	na
	PYGA	F5349	na	na	na	na
KK2T10 (*Spiromyces* sp.)	PYGB	F5315	na	na	na	na
	PYGA	F5967	na	na	na	na
KK2T11b (*Penicillium* sp.)	PYGB	F5316	na	na	na	na
	PYGA	F5968	na	na	na	na
KK2T13b (*Spiromyces* sp.)	PYGB	F5386	na	na	na	na
	PYGA	F5877	na	na	na	na
KK2T14b (*Cladosporium* sp.)	PYGB	F5317	na	na	na	na
	PYGA	F5969	na	na	na	na
KK2T16 (*Phoma* sp.)	PYGB	F5365	na	na	na	na
	PYGA	F5970	na	na	na	na
KK2T17b (*Penicillium* sp.)	PYGB	F5319	na	na	na	na
	PYGA	F5971	na	na	na	na
KK2L1 (*Cladosporium* sp.)	PYGB	F5320	na	na	na	na
	PYGA	F5972	na	na	na	na
KK2L6 (unidentified)	PYGB	F6104	54.5	na	na	na
	PYGA	F5878	na	na	na	na
KK3T2 (*Dreschlera*-like sp.)	PYGB	F5322	na	na	na	na
	PYGA	F5880	na	na	na	na
KK3T4 *(Alternaria* sp.)	PYGB	F5323	na	na	na	na
	PYGA	F5886	na	na	na	na
KK3T8 (*Penicillium* sp.)	PYGB	F5325	na	na	na	na
	PYGA	F5350	96.7	na	72.7	5,403
KK3T15 (*Penicillium* sp.)	PYGB	F5327	na	na	na	na
	PYGA	F5888	na	na	na	na
KK3T18 (*Acremonium* sp.)	PYGB	F5366	na	na	na	na
	PYGA	F5882	na	na	na	na
KK3T21 (*Spiromyces* sp.)	PYGB	F5389	na	na	na	na
	PYGA	F5883	na	na	na	na
KK3T23 (*Penicillium* sp.)	PYGB	F5329	83.9	na	na	4,967
	PYGA	F5890	78.8	na	na	3,177
KK3T25 (*Acremonium* sp.)	PYGB	F5367	na	na	na	na
	PYGA	F5353	na	na	na	na
KK3T26 (*Penicillium* sp.)	PYGB	F5330	69.7	na	na	8,240
	PYGA	F5891	90.0	na	na	3,177
KK3T28 (*Phoma* sp.)	PYGB	F5368	na	na	na	na
	PYGA	F5895	61.8	na	na	na
KK3L1 (*Cladosporium* sp.)	PYGB	F5331	na	na	na	na
	PYGA	F5892	59.4	na	na	na
KK3L2 (*Cladosporium* sp.)	PYGB	F5332	na	na	na	na
	PYGA	F5893	76.2	na	na	na
KK3L13 (unidentified)	PYGB	F5333	na	na	na	na
	PYGA	F5881	88.9	na	na	na
KK3L14 (unidentified)	PYGB	F5334	na	na	na	na
	PYGA	F5973	na	na	na	na

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: growth inhibition (%) against *C. albicans*; D: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

Table 3.6 (cont'd):

Isolates	Media	Extracts	A	B	C	D
KK3L15 *(Spiromyces* sp.)	PYGB	F5390	na	na	na	na
	PYGA	F5884	na	na	na	na
KK 3L21 (unidentified)	PYGB	F5335	na	na	na	na
	PYGA	F5894	54.3	na	na	na
KK4T3 (*Alternaria* sp.)	PYGB	F5336	na	na	na	na
	PYGA	F5857	56.5	na	na	na
KK4T5 (unidentified)	PYGB	F5338	na	na	na	na
	PYGA	F5865	61.2	72.5	na	na
KK4T6 (*Penicillium* sp.)	PYGB	F5339	na	64.3	na	na
	PYGA	F5866	na	na	na	na
KK4T7a (*Alternaria* sp.)	PYGB	F5534	na	na	na	na
	PYGA	F5859	na	na	na	na
KK4T7b (*Penicillium* sp.)	PYGB	F5340	na	na	na	na
	PYGA	F5867	70.6	na	na	6,303
KK4T9 (unidentified)	PYGB	F5341	na	na	na	na
	PYGA	F5910	55.6	na	na	na
KK4T14b (*Penicillium* sp.)	PYGB	F5342	94.5	na	na	<97.5
	PYGA	F5868	95.7	na	na	383
KK4T19 (*Spiromyces* sp.)	PYGB	F5391	na	na	na	na
	PYGA	F5885	na	na	na	na
KK4L2 (*Acremonium* sp.)	PYGB	F5392	na	na	na	na
	PYGA	F5354	na	na	na	na
KK4L4a (*Penicillium* sp.)	PYGB	F5344	53.3	na	na	7,100
	PYGA	F5870	57.2	na	na	na
KK4L4b (*Beauveria bassiana*)	PYGB	F5374	na	na	na	na
	PYGA	F5860	72.3	na	na	na
KK 4L5c (*Penicillium* sp.)	PYGB	F5345	na	na	na	na
	PYGA	F5871	68.6	na	na	na
KK4L7 *(Verticillium* sp.)	PYGB	F5346	na	na	na	na
	PYGA	F5861	101.7	na	na	11,779
KK4L8 (*Penicillium* sp.)	PYGB	F5347	94.6	na	na	375
	PYGA	F5872	94.9	na	na	883
KK8L2 (unidentified)	PYGB	F5537	na	na	na	na
	PYGA	F5875	74.1	na	na	na
KK8L15 (*Paecilomyces* sp.)	PYGB	F5377	57.0	na	na	9,282
	PYGA	F5876	82.4	na	na	12,135
KK8L23 (*Beauveria* sp.)	PYGB	F5574	66.9	na	na	na
	PYGA	F5863	60.2	na	na	na
KK8L24 (*Penicillium* sp.)	PYGB	F5538	58.8	na	na	na
	PYGA	F5965	na	na	na	na
LY1L1 (unidentified)	PYGB	F5555	na	na	na	na
	PYGA	F5974	na	na	na	na
LY1L2 (unidentified)	PYGB	F5369	52.0	na	na	9,010
	PYGA	F5991	63.2	na	na	na
LY1L4 (unidentified)	PYGB	F5557	71.8	na	na	na
	PYGA	F5992	59.9	na	na	na
LY1L5 (*Penicillium* sp.)	PYGB	F5558	69.8	na	na	5,938
	PYGA	F5975	65.2	na	na	5,117

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: growth inhibition (%) against *C. albicans*; D: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

Table 3.6 (cont'd):

Isolates	Media	Extracts	A	B	C	D
LY1L6 (unidentified)	PYGB	F5378	77.6	na	na	11,779
	PYGA	F5976	75.5	na	na	na
LY1L7 (unidentified)	PYGB	F6106	na	na	na	na
	PYGA	F5993	59.9	na	na	na
LY2T1 (unidentified)	PYGB	F5379	na	na	na	na
	PYGA	F5978	na	na	na	na
LY2T3 (unidentified)	PYGB	F5561	71.6	na	na	10,456
	PYGA	F5986	na	na	na	na
LY2T4 (unidentified)	PYGB	F5380	na	na	na	na
	PYGA	F5979	na	na	na	na
LY2L2 (unidentified)	PYGB	F5562	na	na	na	na
	PYGA	F5987	50.9	na	na	4,821
LY2L4 (unidentified)	PYGB	F5564	76.4	na	na	8,240
	PYGA	F5980	na	na	na	na
LY2L5 (unidentified)	PYGB	F5565	64.8	na	na	na
	PYGA	F5981	na	na	na	na
LY2L6 (unidentified)	PYGB	F5566	74.3	na	na	na
	PYGA	F5988	na	na	na	na
LY2L8 (unidentified)	PYGB	F5568	80.0	na	na	8,240
	PYGA	F5989	na	na	na	na
LY2L9 (unidentified)	PYGB	F5371	na	na	na	na
	PYGA	F5982	51.2	na	na	na
LY2L10 (unidentified)	PYGB	F6107	59.3	na	na	na
	PYGA	F5983	95.1	na	na	4,821
LY2L11 (unidentified)	PYGB	F5383	na	na	na	na
	PYGA	F5984	na	na	na	na
LY2L17 (unidentified)	PYGB	F6108	54.2	na	na	na
	PYGA	F5985	67.2	na	na	12,135
LY3T1 (unidentified)	PYGB	F5539	na	na	na	na
	PYGA	F6097	62.2	na	na	na
LY 3T2 (unidentified)	PYGB	F6120	62.2	na	na	na
	PYGA	F6101	63.8	na	na	na
LY3T4a (unidentified)	PYGB	F6118	54.3	na	na	na
	PYGA	F6098	51.5	na	na	na
LY3T8 (unidentified)	PYGB	F6111	na	na	na	na
	PYGA	F5995	57.5	na	na	na
LY3T11 (unidentified)	PYGB	F6119	67.4	na	na	9,563
	PYGA	F6099	na	na	na	na
LY3L1 (unidentified)	PYGB	F5541	71.5	na	na	na
	PYGA	F6100	na	na	na	na
LY3L4 (unidentified)	PYGB	F6112	53.5	na	na	na
	PYGA	F5996	na	na	na	na
LY3L7 (unidentified)	PYGB	F6116	na	na	na	na
	PYGA	F6000	83.6	na	na	na
LY3L12 (unidentified)	PYGB	F6117	69.6	na	na	na
	PYGA	F6096	66.3	na	na	na
LY3L14 (unidentified)	PYGB	F6113	62.2	na	na	na
	PYGA	F5997	69.5	na	na	11,779

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: growth inhibition (%) against *C. albicans*; D: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

Table 3.6 (cont'd):

Isolates	Media	Extracts	A	B	C	D
LY3L16a (unidentified)	PYGB	F6114	69.3	na	na	8,240
	PYGA	F5998	76.1	na	na	9,852
LY3L19 (unidentified)	PYGB	F6115	na	na	na	na
	PYGA	F5999	59.2	na	na	9,852
LY4T1 (unidentified)	PYGB	F5578	79.7	na	na	na
	PYGA	F5905	93.3	na	na	na
LY4T4 (unidentified)	PYGB	F5579	55.7	na	na	na
	PYGA	F5906	72.0	na	na	na
LY4T5 (unidentified)	PYGB	F5580	57.0	na	na	na
	PYGA	F5911	56.9	na	na	5,938
LY4L3 (unidentified)	PYGB	F5545	67.7	na	na	na
	PYGA	F5897	67.3	na	na	na
LY 4L4 (unidentified)	PYGB	F5575	64.0	na	na	na
	PYGA	F5898	98.4	na	na	na
LY 4L7 (*Verticillium* sp.)	PYGB	F5547	63.2	na	na	na
	PYGA	F5900	95.1	na	na	na
LY 4L8a (unidentified)	PYGB	F6109	na	na	na	na
	PYGA	F6102	54.7	na	na	na
LY 4L8b (unidentified)	PYGB	F6110	65.2	na	na	na
	PYGA	F6103	60.1	na	na	na
LY 4L10 (unidentified)	PYGB	F5577	na	na	na	na
	PYGA	F5901	98.6	na	na	na
LY 4L11 (unidentified)	PYGB	F5548	51.5	na	na	na
	PYGA	F5902	na	na	na	na
LY4L13a (unidentified)	PYGB	F5542	na	na	na	na
	PYGA	F5903	101.4	na	na	na
LY4L13b (unidentified)	PYGB	F5543	61.1	na	na	na
	PYGA	F5904	na	na	na	na
LY5T9 (unidentified)	PYGB	F5550	97.2	111.7	na	1,300
	PYGA	F5914	100.5	112.5	na	458
LY5T14 (unidentified)	PYGB	F5551	na	na	na	na
	PYGA	F5915	60.6	na	na	na
LY5T16 (*Scopulariopsis* sp.)	PYGB	F5372	na	na	na	na
	PYGA	F5916	54.5	na	na	na
LY5T24a (unidentified)	PYGB	F5552	50.8	na	na	na
	PYGA	F5917	86.1	na	na	na
LY5L8 (*Aspergillus* sp.)	PYGB	F5373	na	na	na	na
	PYGA	F5919	na	na	na	na

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: growth inhibition (%) against *C. albicans*; D: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

3.4.3 Malaysian fungi

The quick screening assay of the Malaysian fungi showed that 27 of 58 extracts assayed were potentially active (growth inhibition >50%). Of the 27 extracts, 24 showed activity only against P388 cells, two were active against both P388 cells and *B. subtilis* and the remaining extract inhibited only the growth of *B. subtilis*. None of the extracts showed activity against *P. aeruginosa* or *C. albicans*. Of the 26 extracts that inhibited the growth of P388 cells in the quick screening assay, only 11 showed IC_{50}<12,500 ng/mL. The strongest cytotoxic activity was shown by two extracts, namely F6448 and F6451 (both with IC_{50}3,886 ng/mL). The results of bioactivity are shown in Table 3.7.

Table 3.7: Bioactivity of Malaysian fungi.

Isolates	Media	Extracts	A	B	C
PR1T2 (unidentified)	F6401	PYGB	51.3	na	4,938
PR1T3 (unidentified)	F6402	PYGB	na	na	na
PR1T4 (unidentified)	F6403	PYGA	na	na	na
PR1L3 (unidentified)	F6404	PYGB	59.0	na	na
PR2T2 (unidentified)	F6406	PYGA	82.4	na	4,126
PR2T4 (unidentified)	F6407	PYGB	74.3	na	na
PR2T10 (unidentified)	F6408	PYGB	93.4	na	5,911
PR2L6 (unidentified)	F6409	PYGB	50.4	na	na
PR3T3 (unidentified)	F6410	PYGA	na	na	na
PR3T11 (unidentified)	F6411	PYGB	na	na	na
PR3T12 (*Trichoderma* sp.)	F6412	PYGB	79.9	na	7,512
PR3T13 (unidentified)	F6413	PYGB	na	na	na
PR3T19 (unidentified)	F6414	PYGB	68.9	na	na
PR3L8 (*Xylaria* sp.)	F6415	PYGB	92.0	na	4,004
PR3L15 (unidentified)	F6416	PYGA	na	na	na
PR3L16 (unidentified)	F6417	PYGA	na	na	na
PR3L17 (unidentified)	F6418	PYGB	na	na	na
PR4T2 (unidentified)	F6419	PYGB	na	na	na
PR4T3 (unidentified)	F6420	PYGB	na	na	na
PR4T4 (unidentified)	F6421	PYGB	na	na	na
PR4T5 (unidentified)	F6422	PYGB	na	na	na
PR4T7 (unidentified)	F6423	PYGA	60.3	100.6	na
PR4T8 (unidentified)	F6424	PYGB	64.0	na	na
PR4T9 (unidentified)	F6425	PYGB	na	na	na
PR5T2 (unidentified)	F6426	PYGB	78.9	na	12,133
PR5T3 (unidentified)	F6427	PYGA	na	na	na
PR5T4 (unidentified)	F6428	PYGB	53.8	na	na
PR5T6 (unidentified)	F6429	PYGB	na	na	na
PR5L9 (*Paecilomyces* sp.)	F6430	PYGB	66.0	na	10,445
PR7T2 (unidentified)	F6432	PYGA	na	na	na
PR7T3 (unidentified)	F6433	PYGA	58.5	na	na
PR8L5 (unidentified)	F6434	PYGA	na	na	na
PR8L8 (unidentified)	F6435	PYGB	62.5	na	na
PR9T5 (unidentified)	F6436	PYGA	na	na	na
PR9L1 (unidentified)	F6437	PYGB	na	na	na
PR10T2 (*Paecilomyces* sp.)	F6439	PYGA	86.8	na	12,881(*)
PR10T8 (unidentified)	F6440	PYGB	66.2	na	na
PR10L10a (unidentified)	F6441	PYGA	na	na	na
PR10L10b (unidentified)	F6443	PYGB	na	na	na
PR11T4 (unidentified)	F6444	PYGB	59.9	na	8,726
PR12T1 (unidentified)	F6445	PYGB	na	na	na
PR13T1 (unidentified)	F6446	PYGA	53.6	na	na
PR13T2 (unidentified)	F6447	PYGB	55.7	na	na
PR13T2 (unidentified)	F6448	PYGA	92.3	na	3,886
PR13T6 (unidentified)	F6449	PYGB	na	na	na

A: growth inhibition (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL; (*) extract F6439 (IC_{50}>12,500 ng/mL) was selected due to interesting chemical properties (see **Chapter Six** – *A Malaysian Paecilomyces sp. (PR10T2)*).

Table 3.7 (cont'd):

Isolates	Media	Extracts	A	B	C
PR14T8 (unidentified)	F6450	PYGA	na	na	na
PR17L2 (unidentified)	F6451	PYGB	92.4	na	3,886
PR17L5 (unidentified)	F6452	PYGB	na	na	na
PP1T2 (unidentified)	F6453	PYGB	91.3	na	na
PP1T3 (unidentified)	F6454	PYGB	na	na	na
PP1T4 (unidentified)	F6455	PYGB	na	na	na
PP1T7 (unidentified)	F6456	PYGB	na	na	na
PP2L4 (unidentified)	F6457	PYGB	88.2	na	12,500
PP2L5 (unidentified)	F6458	PYGB	na	na	na
PP4L3 (unidentified)	F6459	PYGB	na	109.7	na
PP4L6 (unidentified)	F6460	PYGB	93.0	104.6	na
PP4T1 (unidentified)	F6461	PYGA	na	na	na
PP4T2 (unidentified)	F6462	PYGB	na	na	na

A: growth inhibiton (%) against P388 cells; B: growth inhibition (%) against *B. subtilis*; C: IC_{50} (ng/mL) against P388 cells; na: growth inhibition <50% or IC_{50}>12,500 ng/mL.

3.5 HPLC screening of bioactive extracts

HPLC screening for significant peaks provides a basis for selection of extracts for further investigation. The presence of significant amounts of metabolites was indicated by both the UV and ELSD traces (>10% of both the base peaks in the HPLC chromatogram). The HPLC-ELSD profile was used to indicate the amount of these metabolites present in the extract since HPLC-ELSD is generally considered a quasi-universal detector of the quantity of an analyte in a sample (Higgs *et al*., 2001). Fungal extracts that contained significant peak(s) were further screened to establish if any of the peak(s) match in both UV chromophores and retention time (R_t) with known compounds within the in-house HPLC-UV/R_t library database. If all significant peaks resulted in 'known hits', further work on that extract was discontinued. Details of the procedures are discussed in **Chapter Two** (*Experimental*).

A total of 54 extracts that showed IC_{50}<12,500 ng/mL in the P388 assay or inhibited >50% of the growth of *B. subtilis* or *C. albicans* in the antimicrobial quick screen assay were subjected to HPLC screening. Of the total 54 extracts examined, only eight extracts were chosen for further investigation based on good cytotoxicity and significant traces of metabolites. An extract (F6439; IC_{50}12,881 ng/mL) was also further investigated due to interesting chemical properties. These nine extracts were obtained from isolate LA3L2 (see **Chapter Five** - *Secondary metabolites from marine-derived Streptomyces sp. (LA3L2)*), isolate PR10T2 (see **Chapter Six** - *A Malaysian Paecilomyces sp. (PR10T2)*), isolates LY1L5 and KK3T23 (see **Chapter Four** - *New Zealand Penicillium spp.* and **Chapter Seven** - *Application of rapid techniques for chemical characterization of metabolites*) and isolates LA3L1, KK3T8, KK4T14b, LA5L4 and PR5L9 (see **Chapter Seven** - *Application of rapid techniques for chemical characterization of metabolites*).

Some examples of bioactive extracts that were not selected for further study because of the presence of known and/or insignificant quantity of metabolites are given below.

Example 1:

Six extracts were not investigated further due to the presence of known metabolites are listed in Table 3.8. An example is presented below using the results obtained from extract F6451.

Table 3.8: Dereplication of metabolites from fungal extracts using the in-house HPLC-UV/R_t library database.

Metabolites	Isolates	Extracts (media)
dihydrosterigmatocystin	PR17L2	F6451 (PYGB)
indole-3-carboxylic acid	LY5T9	F5550 (PYGB) , F5914 (PYGA)
Meleagrine	PR2T2	F6406 (PYGA)
mycophenolic acid	KK4L8	F5347 (PYGB), F5872 (PYGA)

The HPLC screening of F6451 showed one significant peak eluting at 16.8 min (Figure 3.2). A search of the HPLC-UV/R_t library database for this peak showed matches in both UV chromophores and R_t to the known compound, dihydrosterigmatocystin (see Figure 3.3). Since the main significant peak resulted in an exact match with the known compound further work was not carried out.

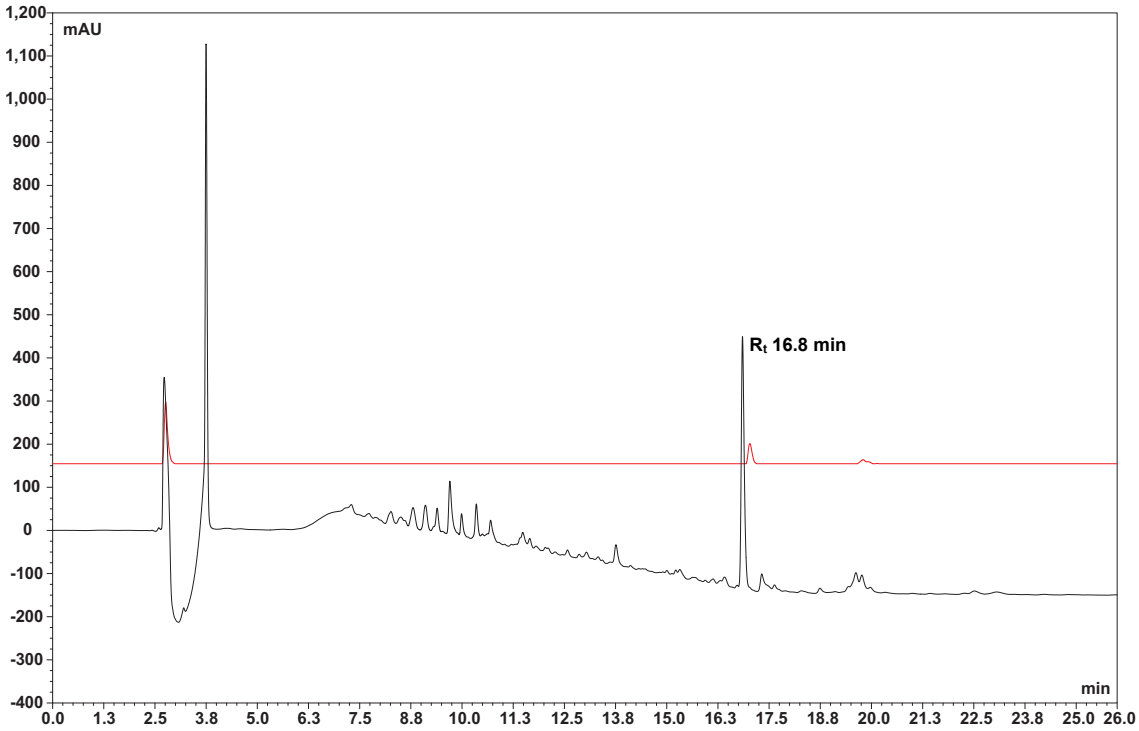


Figure 3.2: HPLC chromatogram of F6451 showing overlay of ELSD detection (top) for the main peak eluted at 16.8 min.

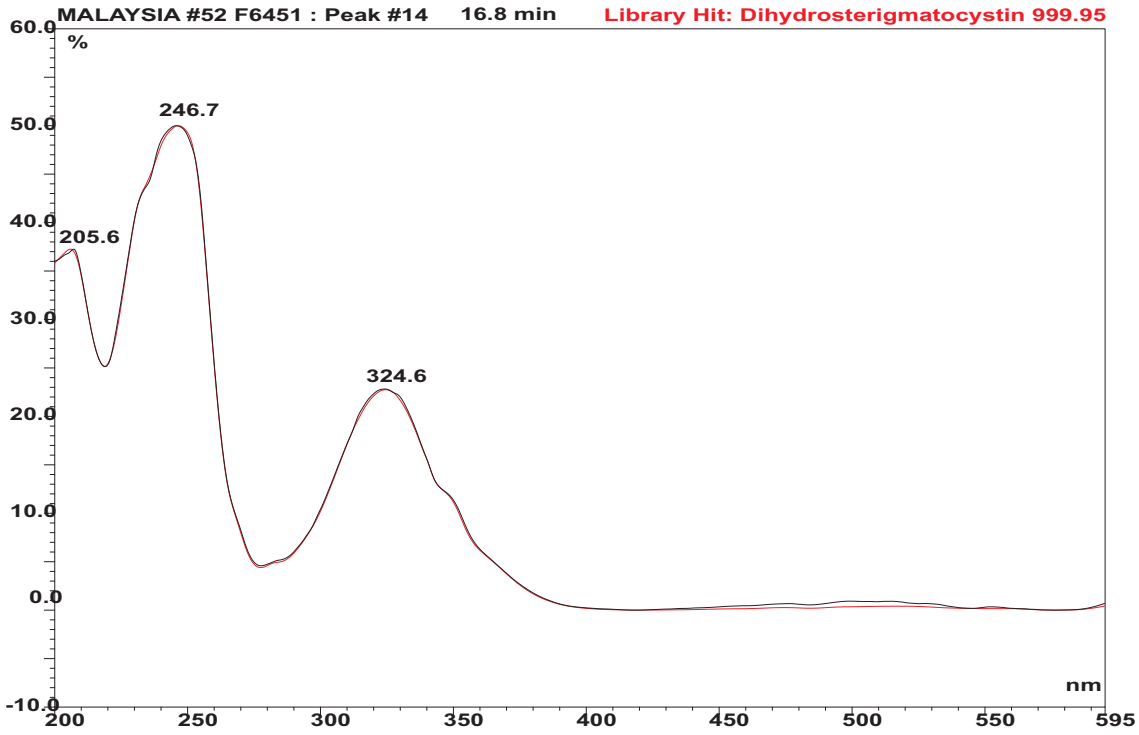


Figure 3.3: UV spectrum of the main peak eluted at 16.8 min (black) showing the UV library hit (red) with the known dihydrosterigmatocystin in the HPLC-UV/R_t library database.

Example 2:

Examples of extract that were not chosen due to insignificant traces of metabolites are shown by using results obtained from isolates KK3T26 and LA2T4.

a) Isolate KK3T26 (F5891 vs. F5330)

Extracts F5891 and F5330 were obtained from cultures of isolate KK3T26 grown on agar medium (PYGA) and broth medium (PYGB), respectively. Both extracts showed cytotoxicity but were not active in the antimicrobial assays. Extract F5891 was more cytotoxic (IC_{50}3,177 ng/mL) than F5330 (IC_{50}8,240 ng/mL). HPLC screening showed that neither extract F5891 or F5330 produced significant amounts of metabolites as indicated by ELSD traces (Figure 3.4). These extracts were not further investigated.

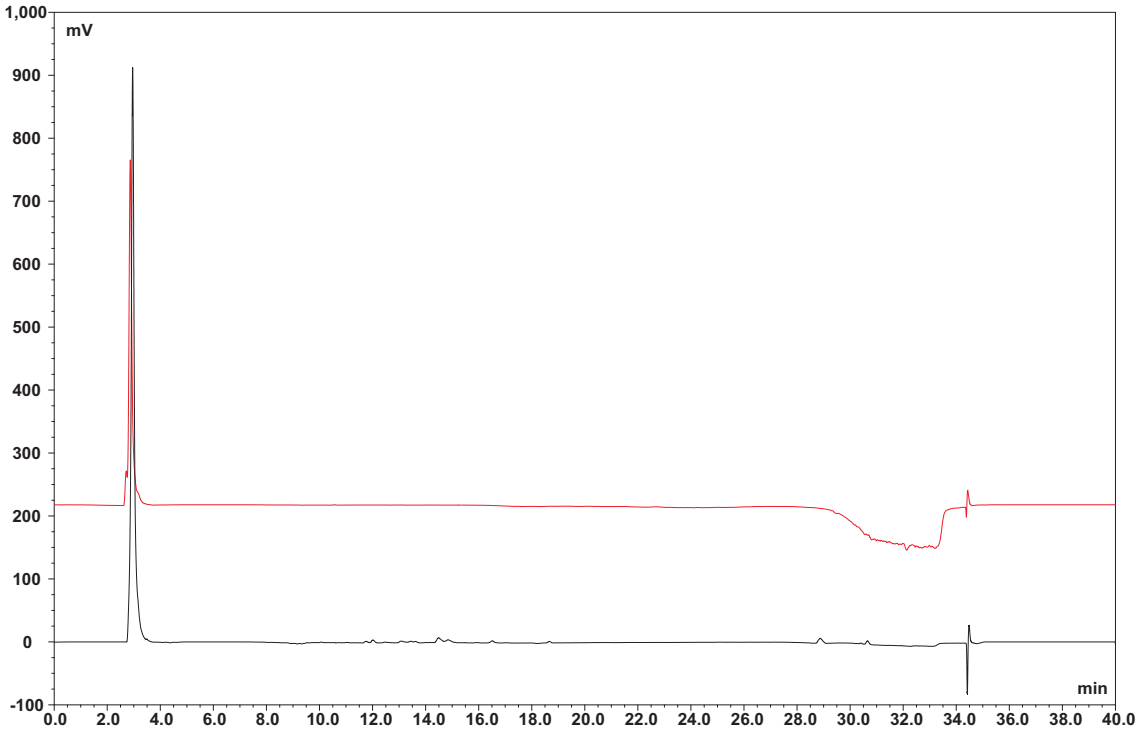


Figure 3.4: HPLC chromatogram showing ELSD traces of crude extracts of isolate KK3T26 obtained from agar medium (F5891 - bottom) in comparison with broth medium (F5330 - top).

b) Isolate LA2T4 (F5954)

Extract F5954 (IC_{50}5,938 ng/mL) was obtained from cultures of isolate LA2T4 grown on agar medium (SCA). HPLC-PDA detected six metabolites with significant UV chromophores (see Figure 3.5). The HPLC-ELSD profile indicated that the traces of all six metabolites were <10% of the ELSD base peak. Although the UV chromophores of these peaks were significant, the HPLC-ELSD profile showed otherwise and this extract therefore, was not pursued further.

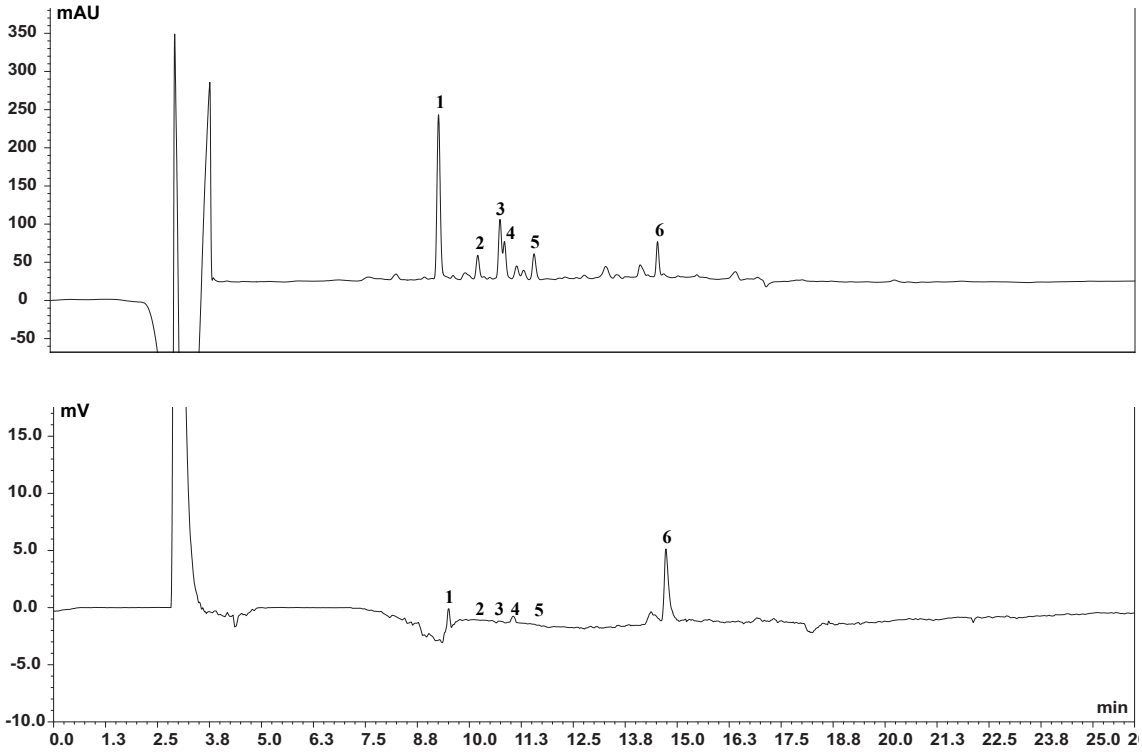


Figure 3.5: HPLC chromatogram of F5954 showing six metabolites as detected by UV (top) and ELSD (bottom). The peaks showing traces >10% of the UV base peak but none of them showing traces >10% of the ELSD base peak.

Example 3:

Some isolates produced similar metabolites when grown on both agar and broth media, but differed in the amount of metabolite produced. Extracts that produced greater quantitites of metabolites were chosen for further investigation. An example is shown in extracts F5956 and F5934 obtained from isolate LA3L1.

a) Isolate LA3L1 (F5956 vs. F5934)

Extracts F5956 and F5934 were obtained from cultures of isolate LA3L1 grown on agar medium (SCA) and broth medium (SCB), respectively. Both extracts showed significant cytotoxicity (IC_{50}<97.5 ng/mL) and were active against *B. subtilis* (>99% inhibition). HPLC screening showed that extracts F5956 and F5934 produced similar metabolites (Figure 3.6). Extract F5956, however, produced larger ELSD traces of peaks 1, 2 and 3 compared to F5934. In this case, only F5956 was chosen for further investigation.

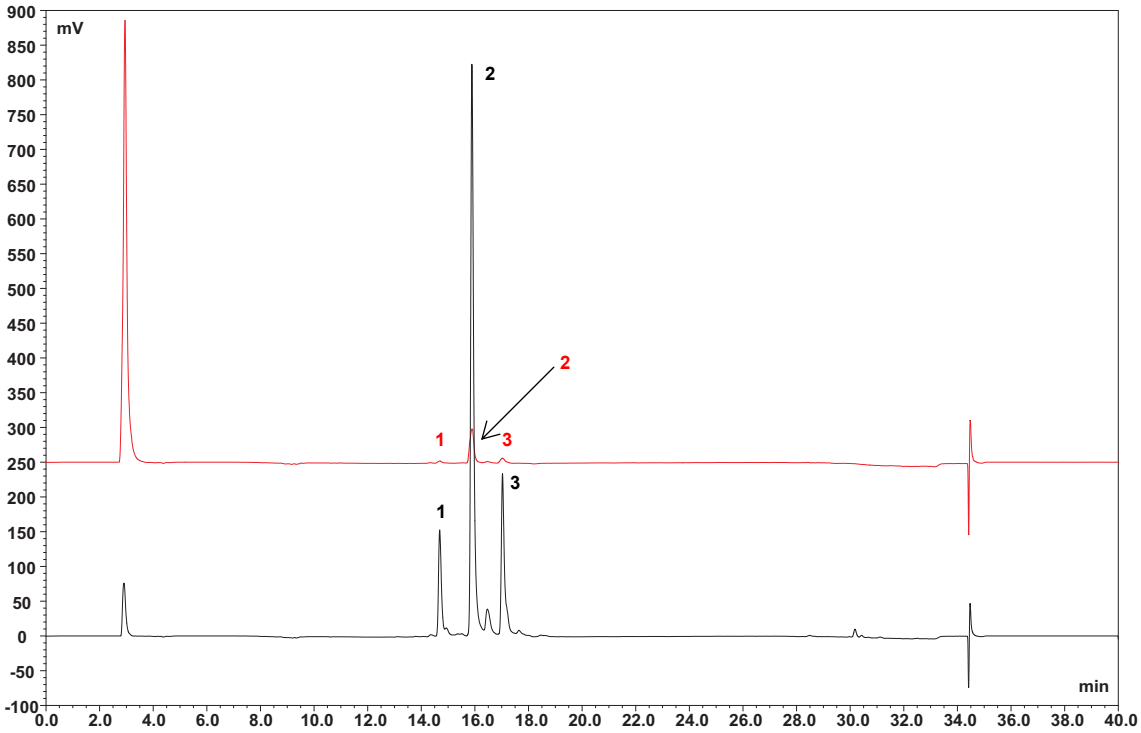


Figure 3.6: HPLC chromatogram showing ELSD traces of crude extracts of isolate LA3L1 obtained from agar medium (F5956 - bottom) in comparison with broth medium (F5934 - top).

3.6 Discussion

This study adopted the isolation technique of Namikoshi *et al.* (2002) for both the tissue and liquid portions of the investigated marine invertebrates. This technique enabled the isolation of many isolates of actinomycetes from the tissue portions of four sponges (K2, K3, K4 and L2), however, none were isolated from the liquid portions. Two isolates were isolated from liquid portions of a tunicate (L3) and none from its tissue portions, while the tissue and liquid portions of another sponge (L5) yielded four and five isolates, respectively. Many fungi could be isolated from tissue and liquid portions of the invertebrates. It is interesting to note that a larger percentage of *Penicillia* were isolated from New Zealand samples than was seen from invertebrates from Malaysia. In light of the world-wide distribution of *Penicillia* it is unlikely that there is a selectivity of association from New Zealand samples and a more likely explanation is that of time that the samples were in storage before isolation was attempted. In the case of Malaysian samples, they were processed immediately after collection but those from New Zealand were stored for up to three days at -20ºC which may have acted as a selective mechanism.

The results from the quick screen assay showed that of 266 extracts assayed, 146 (55%) were potentially active. Of the 146 extracts, 136 (93%) were active only against P388 cells; seven (5%) against both P388 cells and *B. subtilis*, one (1%) against both P388 cells and *C. albicans* and two (1%) against only *B. subtilis*. Of the 144 extracts that were potentially active in the P388 quick screen assay, only 52 (36%) inhibited 50% of the growth of P388 cells at a concentration of <12,500 ng/mL (IC_{50}<12,500 ng/mL). None of the extracts assayed inhibited the growth of *P. aeruginosa*. The actinomycetes screened, mostly *Streptomyces* spp. showed good cytotoxicity. The lack of finding bioactivity in some isolates such as *Acremonium* and *Aspergillus* is perhaps surprising because members of these genera are prolific producers of secondary metabolites, many of them cytotoxic (Bugni and Ireland, 2004; Ebel, 2006).

HPLC screening showed that some of the extracts obtained from agar and/or broth media showed variations in the production of metabolites. The cytotoxicity of New Zealand actinomycetes was dominated by extracts that originated from agar culture medium - six of eight isolates showed activity only when cultured on SCA medium. Of 24 New Zealand fungal isolates that produced cytotoxic extracts, nine were active only when cultured on PYGA medium, seven were active only when cultured on PYGB medium, and the remaining eight isolates were active when cultured on both media. As for the Malaysian fungal isolate, only one isolate that was cultured on both media showed higher cytotoxicity when cultured on PYGA medium. These observations cannot be considered significant since not all Malaysian samples were cultured on both PYGA and PYGB media. Differences in solid and liquid media in the production of metabolites has been noted by many workers (Espinosa and Webb, 2003; Gonzalez *et al*., 2003) however, solid media is difficult in commercial situations and in nearly all cases liquid fermentation is used.

Chapter 4

New Zealand *Penicillium* spp.

4.1 Introduction

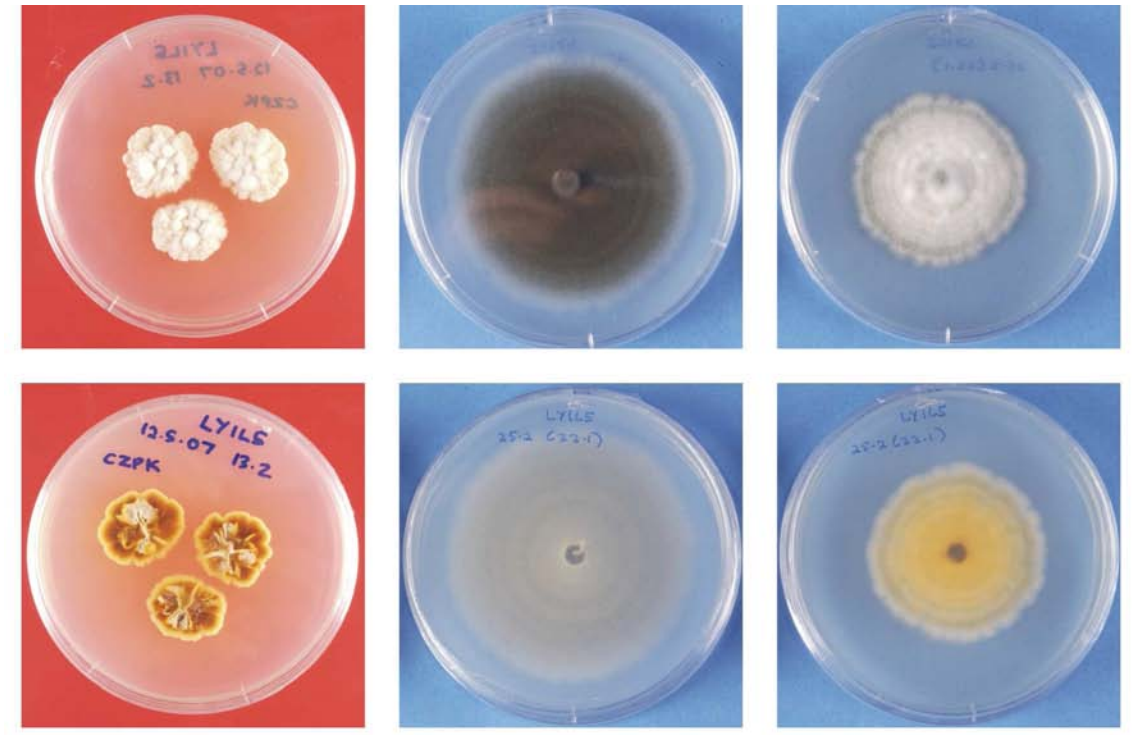
Marine-derived fungi may be defined as these fungi – be they obligate or facultatively marine – that are isolated from the marine environment (Kohlmeyer and Kohlmeyer, 1979). It is not always clear, however, whether all these isolates actually grow in this saline environment or are itinerants. For those who are active in the marine world then osmoregulatory mechanisms must be in place. Osmoregulation is energetically costly and it has been postulated that such fungi exhibit decreased amounts or rate of secondary metabolite production in the presence of salt (Bugni and Ireland, 2004). Salt-dependent strains of marine-derived fungi for metabolite production have been described for *Penicillium* spp. (Bugni and Ireland, 2004). Some studies have indicated that production of metabolites from marine-derived fungi is sensitive to seawater concentration (Miller and Savard, 1989; Masuma *et al*., 2001). This would have implications in drug discovery programmes using marine-derived organisms. In this study, two isolates of marine-derived *Penicillium* spp. showing cytotoxicity were assessed for their tolerance to salinity (0, 2, 4, 6, 8 and 10% sea salt concentration) in respect to their growth and cytotoxic metabolite production.

4.2 *Penicillium* sp. (LY1L5)

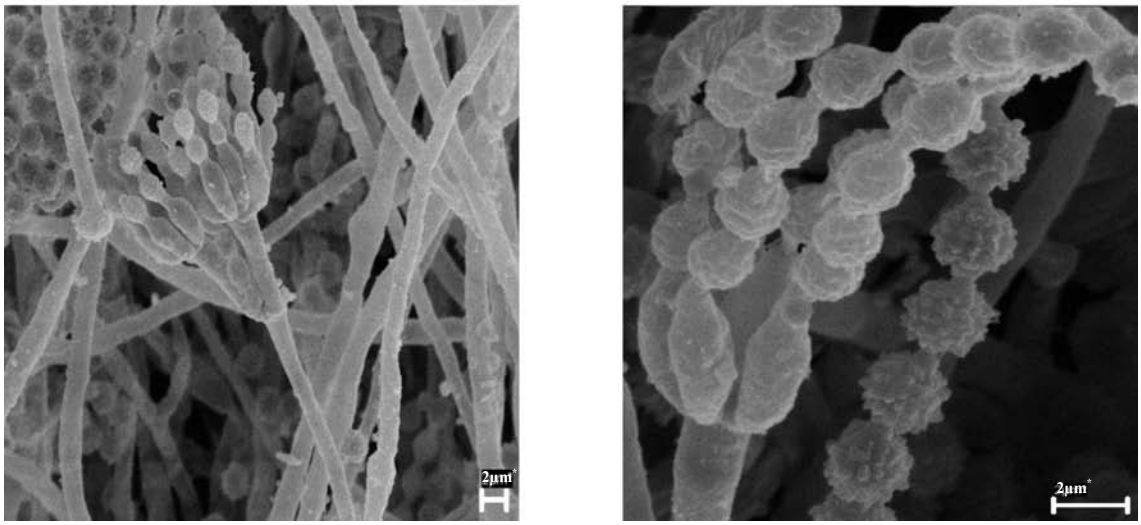
Penicillium sp. (LY1L5) was isolated from liquid portions of a tunicate collected in Lyttleton Harbour in 2004.

4.2.1 Cultural characteristics and morphology

Morphological characteristics of isolate LY1L5 are shown in Figure 4.1. Cultures on Czapek medium 20 – 25 mm diameter on ten days at 25ºC, mycelium white, reverse yellow or brownish. Cultures on PYGA (4% sea salt) medium 30 - 35 mm diameter after ten days at 25ºC, mycelium black, reverse greyish. Cultures on PYGA (0% sea salt) medium 25 - 30 mm diameter after ten days at 25ºC, mycelium white, reverse yellow or brownish. Stipes and metulae with smooth walls, conidiophores with two-stage-branched, biverticillate on PYGA (4% sea salt) medium. Phialides four to eight per metulae, ampulliform, 4.0 – 6.0 x 2.0 – 2.5 μm; conidia spheroidal, spinose, 1.5 – 2.0 μm diameter, in chains with small connectives between the conidia.



(a) (b) (c)

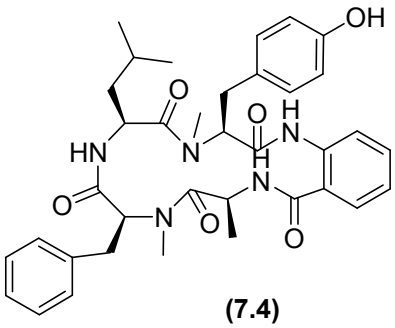


(d) (e)

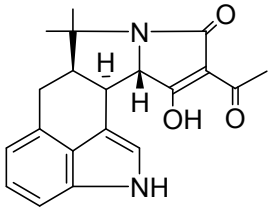
Figure 4.1: Colony and morphological characteristics of *Penicillium* sp. (LY1L5); (a) colonies on Czapek medium at 25ºC, 10 days; (b) colonies on PYGA (4% sea salt) medium at 25ºC, 30 days; (c) colonies on PYGA (0% sea salt) medium at 25ºC, 30 days; (d) – (e) SEM of conidiophores and conidia on PYGA (4% sea salt) medium at 25ºC, 10 days (d) conidiophores; (e) conidia.

4.2.2 Metabolite profile

Penicillium sp. (LY1L5) produced both cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**) (see **Chapter Seven** - *Application of rapid techniques for chemical characterization of metabolites*). Its metabolite profile was compared with those *Penicillium* spp. reported to produce cycloaspeptide A (**7.4**) (five species) and α-cyclopiazonic acid (**7.5**) (six species) (see Table 4.1). The comparison showed that none of the 11 *Penicillium* spp. produced both cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**) (Frisvad *et al*., 2004; Frisvad *et al*., 2006).



(7.4)



(7.5)

Table 4.1: Comparison of secondary metabolite profile of *Penicillium* sp. (LY1L5) with the reported *Penicillium* spp. producing cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**).

***Penicillium* spp.**	**cycloaspeptide A (7.4)**	**α-cyclopiazonic acid (7.5)**
Penicillium* sp. (LY1L5*)	+	+
P. jamesonlandense	+	
P. ribeum	+	
P. lanosum	+	
P. soppii	+	
P. algidum	+	
P. camemberti		+
P. commune		+
P. dipodomyicola		+
P. griseofulvum		+
P. patulum		+
P. palitans		+

4.3 *Penicillium* sp. (KK3T23)

Penicillium sp. (KK3T23) was isolated from tissue portions of a sponge collected off Kaikoura in 2004.

4.3.1 Cultural characteristics and morphology

Morphological characteristics of isolate are shown in Figure 4.2. Cultures on Czapek medium 30 – 35 mm diameter on ten days at 25ºC, mycelium greenish yellow, reverse pale brown. Cultures on PYGA (4% sea salt) medium 45 - 50 mm diameter on ten days at 25ºC, mycelium green, reverse pale brown. Cultures on PYGA (0% sea salt) medium 35 - 40 mm diameter on ten days at 25ºC, mycelium green, reverse pale brown. Stipes bearing terminal penicilli, typically terverticillate on PYGA (4% sea salt) medium. Rami one to two per penicillus, 12.0 – 14.0 x 3.0 – 3.5 µm; metulae 7.5 – 9.0 x 2.5 – 3.5 µm; phialides four to eight per metulae, ampulliform, 7.0 – 8.0 x 2.0 – 3.0 µm; conidia subspheroidal to ellipsoidal, 3.0 – 4.0 x 3.0 – 3.5 µm diameter, with smooth walls, borne in irregular chains.

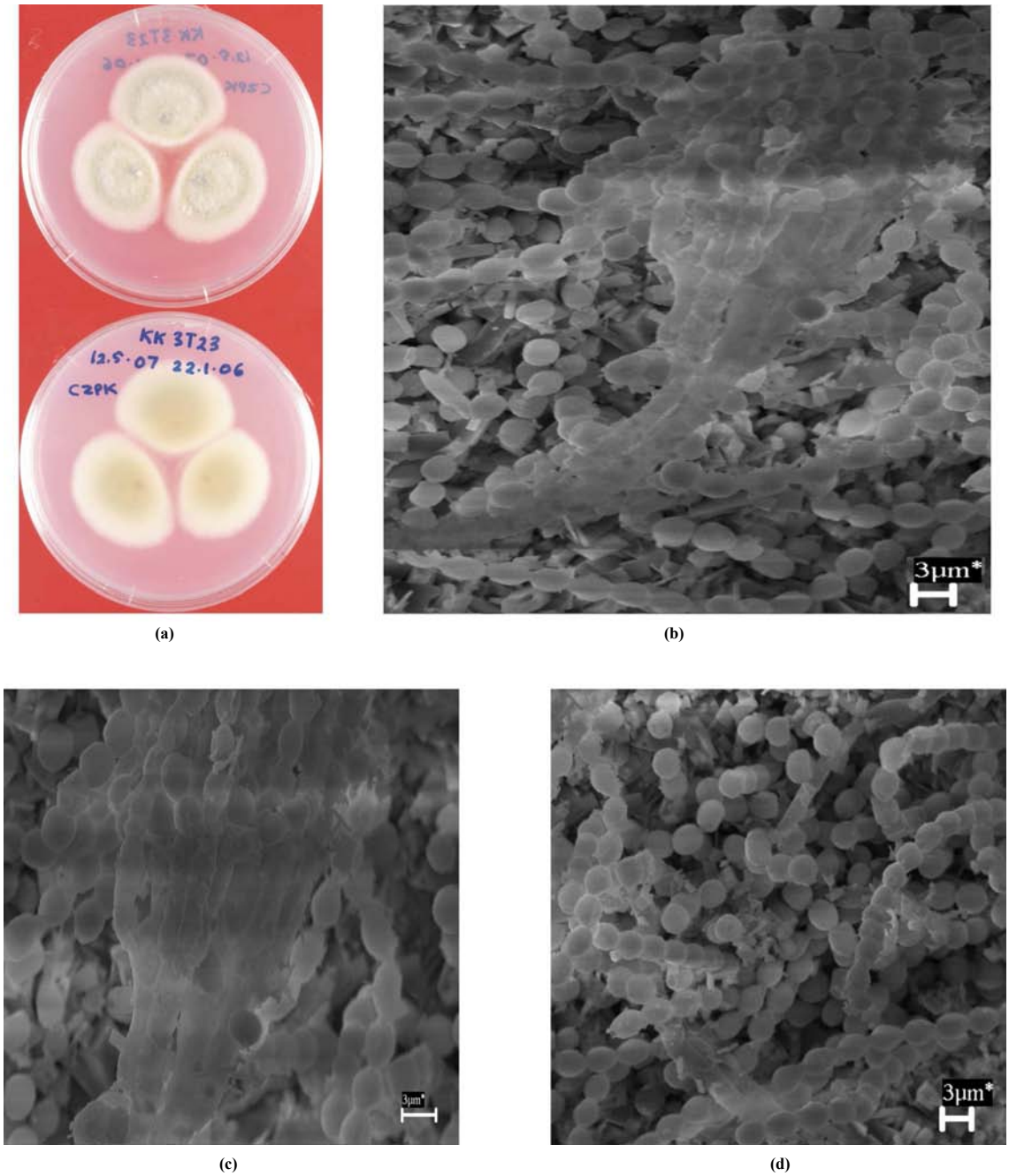
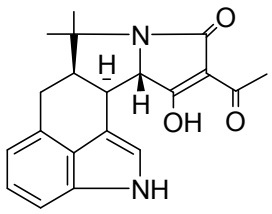


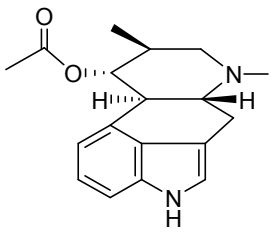
Figure 4.2: Colony and morphological characteristics of *Penicillium* sp. (KK3T23); (a) colonies on Czapek medium at 25ºC, 10 days; (b) – (d) SEM of conidiophores and conidia on PYGA (4% sea salt) medium at 25ºC, 10 days; (b) conidiophores; (c) phialides; (d) conidia.

4.3.2 Metabolite profile

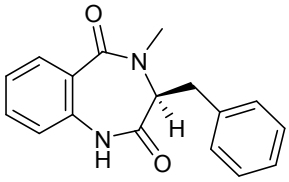
Penicillium sp. (KK3T23) produced α-cyclopiazonic acid (**7.5**), roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**) (see **Chapter Seven** - *Application of rapid techniques for chemical characterization of metabolites*). Its metabolite profile was compared with those *Penicillium* spp. reported to produce α-cyclopiazonic acid (**7.5**), roquefortine A (**7.6**), cyclopeptin (**7.7**), viridicatin (**7.8**) and (see Table 4.2). The comparison showed that only *P. palitans* produced three of the metabolites produced by isolate KK3T23; α-cyclopiazonic acid (**7.5**), cyclopeptin (**7.7**) and viridicatin (**7.8**).



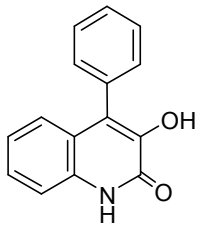
(7.5)



(7.6)



(7.7)



(7.8)

Table 4.2: Comparison of secondary metabolite profile of *Penicillium* sp. (KK3T23) with the reported *Penicillium* spp. producing α-cyclopiazonic acid (**7.5**), roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**).

***Penicillium* spp.**	**Secondary metabolites[a]**			
	α-cyclopiazonic acid (7.5)	**roquefortine A* (7.6)**	**cyclopeptin (7.7)**	**viridicatin (7.8)**
***Penicillium* sp. (KK3T23)**	+	+	+	+
P. camemberti	+	*		
P. commune	+	*		
P. dipidomyicola	+	*		
P. griseofulvum	+	*		
P. patulum	+	*		
P. palitans	+	*	+	+
P. albocoremium		*	+	+
P. allii		*	+	+
P. caseifulvum		*	+	+
P. commune		*	+	+
P. crustosum		*	+	+
P. discolor		*	+	+
P. echinulatum		*	+	+
P. freii		*	+	+
P. hirsutum		*	+	+
P. neoechinulatum		*	+	+
P. polonicum		*	+	+
P. radicicola		*	+	+
P. solitum		*	+	+
P. tulipae		*	+	+
P. venetum		*	+	+
P. vulpinum		*	+	+

[a] results obtained from Frisvad *et al*., 2004; * not reported in Frisvad *et al*., 2004.

4.4 Effect of salinity on growth and production of metabolites

The original cultivation conditions (PYGA medium, temperature 25°C, pH 7.0±0.2 and 4% sea salt) that produced the cytotoxic activity were used as a basis to study the response of salinity to growth and production of metabolites in *Penicillium* sp. (LY1L5) and *Penicillium* sp. (KK3T23). Salinity in this study is expressed as sea salt concentration (%) incorporated in the media. A salinity range of 0, 2, 4, 6, 8 and 10% (PYGA medium; pH 7.0±0.2) was used. Cultures were incubated at 25°C and the colony diameter (three replicates) was measured every 2 days for 30 days. Cultures were harvested after 30 days incubation and extracted. The extracts (1 mg/mL) were assayed for cytotoxicity against P388 cells and their metabolites production assessed by HPLC. The presence of cycloaspeptide A (**7.4**), α-cyclopiazonic acid (**7.5**), roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**) was confirmed by their UV characteristics as described in **Chapter Seven** (*Application of rapid techniques for chemical characterization of metabolites*). Details of the procedures are described in **Chapter Two** (*Experimental*).

4.4.1 *Penicillium* sp. (LY1L5)

The growth of *Penicillium* sp. (LY1L5) was enhanced in the presence of salt (Figure 4.3), but the growth decreased at 10% salinity. Growth rate declined after 18 – 20 days incubation. Cytotoxicity of extracts is shown in Table 4.3. This isolate showed better IC_{50} values at salinity of 4 and 6%, but was not active in the absence of salt.

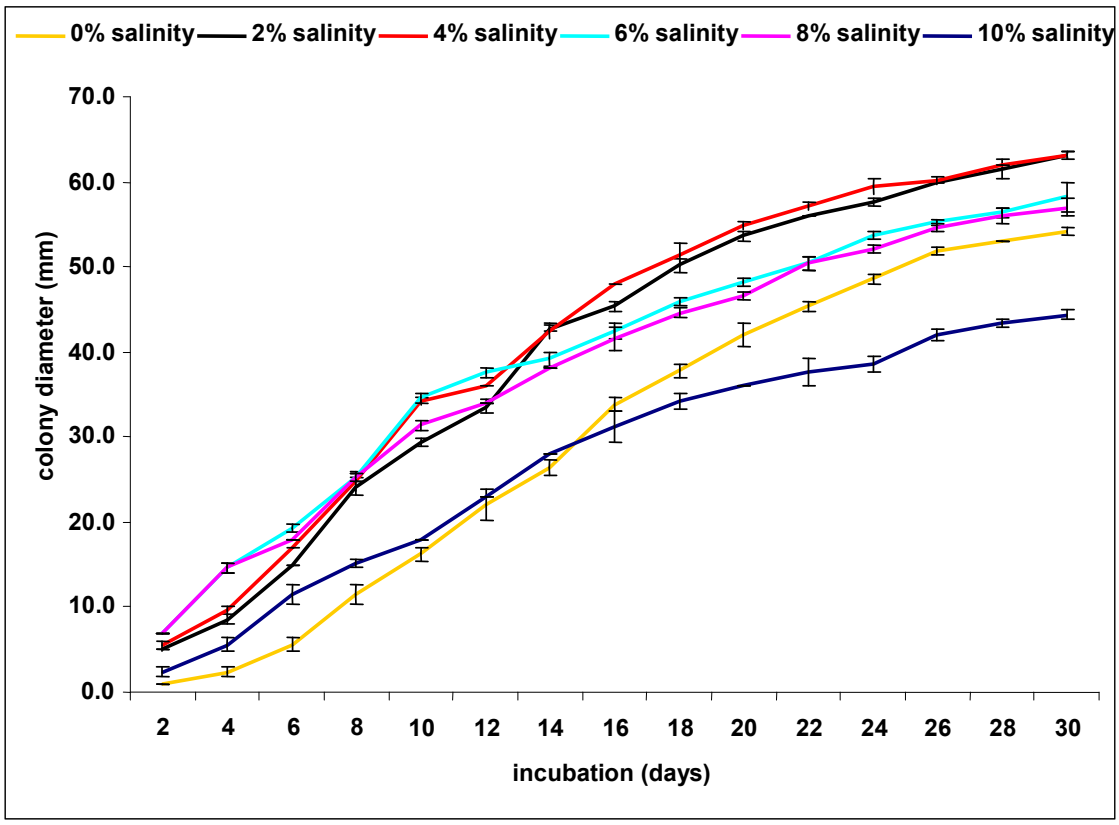


Figure 4.3: Effect of salinity on growth of *Penicillium* sp. (LY1L5) on PYGA medium (pH 7.0±0.2; 25°C).

Table 4.3: Effect of salinity on cytotoxicity of *Penicillium* sp. (LY1L5) on PYGA medium (pH 7.0±0.2; 25°C; 30 days).

Salinity (%)	Cytotoxicity against P388 cells	
	Extracts	IC_{50} (ng/mL)
0	F5975-A	>12,500
2	F5975-B	7,976
4	F5975-C	6,664
6	F5975-D	6,664
8	F5975-E	7,741
10	F5975-F	9,837

The ELSD traces of all six extracts are shown in Figure 4.4. All extracts showed traces of cycloaspeptide A (**7.4**). Traces of the cytotoxic α-cyclopiazonic acid (**7.5**) were detected only in five active extracts (F5975-B, F5975-C, F5975-D, F5975-E and F5975-F) but none in the inactive extract (F5975-A).

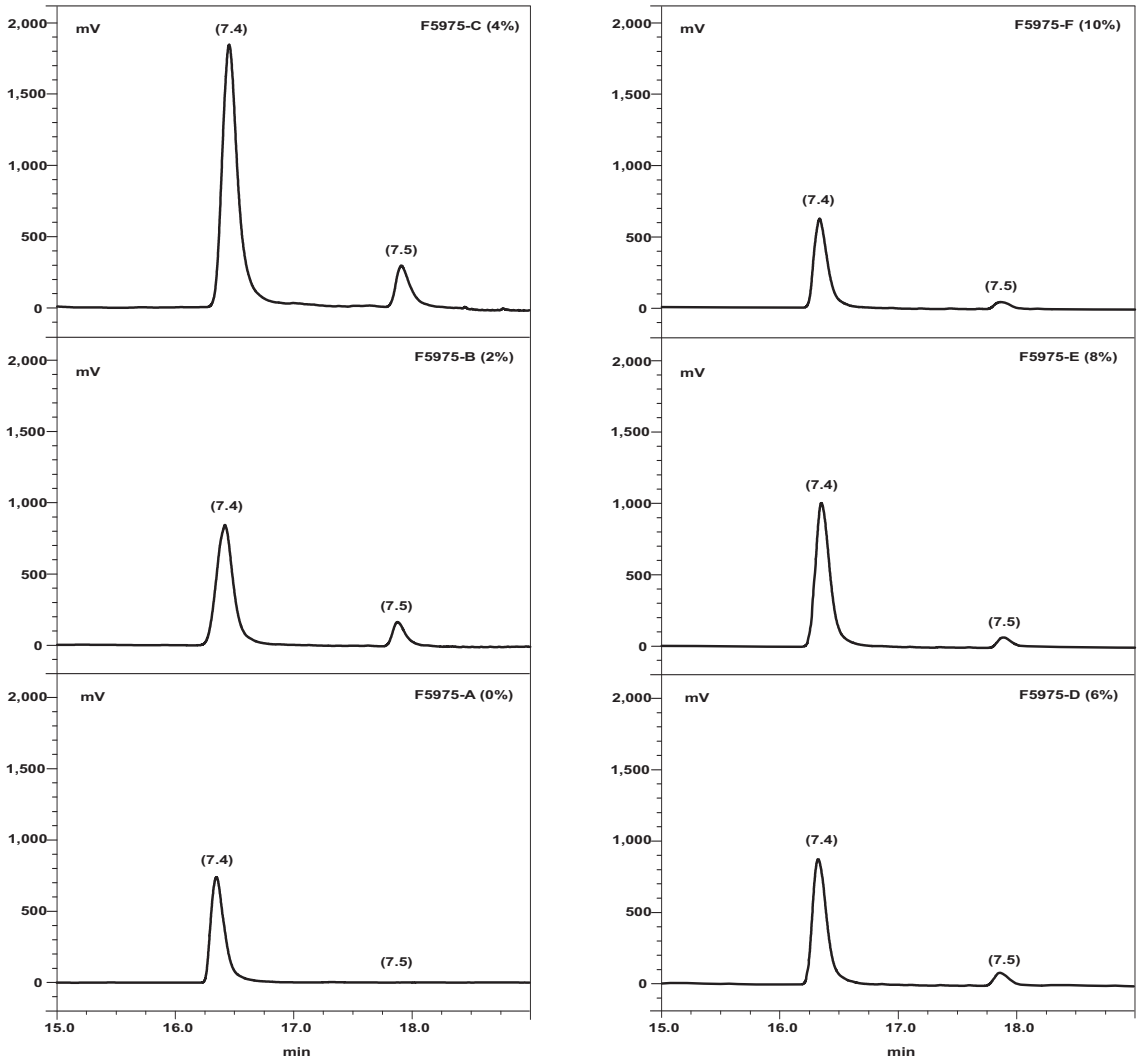


Figure 4.4: ELSD traces of cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**) from extracts of *Penicillium* sp. (LY1L5) cultured on PYGA medium (pH 7.0±0.2; 25°C; 30 days) at salinity of 0, 2, 4, 6, 8 and 10%.

4.4.2 *Penicillium* sp. (KK3T23)

The growth of *Penicillium* sp. (KK3T23) was enhanced in the presence of salt (Figure 4.5) up to a level of 6% but growth decreased at levels higher than this. Growth rate declined after 16 – 18 days of incubation. Cytotoxicity of the extracts is shown in Table 4.4. Maximum salinity was observed at 4% salinity and no activity was recorded at 8 or 10% salinity.

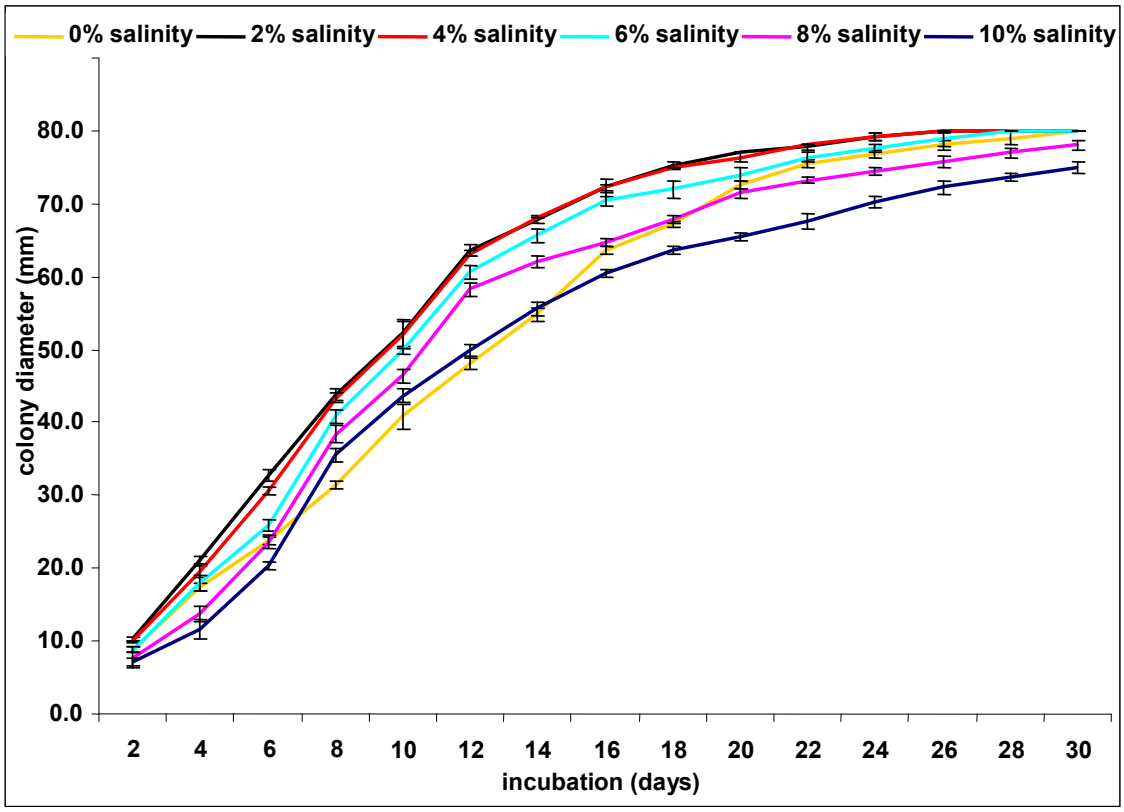


Figure 4.5: Effect of salinity on growth of *Penicillium* sp. (KK3T23) on PYGA medium (pH 7.0±0.2; 25°C).

Table 4.4: Effect of salinity on cytotoxicity of *Penicillium* sp. (KK3T23) on PYGA medium (pH 7.0±0.2; 25°C; 30 days).

Salinity (%)	**Cytotoxicity against P388 cells**	
	Extracts	**IC_{50} (ng/mL)**
0	F5890-A	12,133
2	F5890-B	10,445
4	F5890-C	3,177
6	F5890-D	6,664
8	F5890-E	>12,500
10	F5890-F	>12,500

The ELSD traces of all six extracts are shown in Figure 4.6. Four cytotoxic extracts (F5890-A, F5890-B, F5890-C and F5890-D) contained traces of the cytotoxic α-cyclopiazonic acid (**7.5**). Two extracts F5890-E and F5890-F, that were inactive in the P388 assay did not contain α-cyclopiazonic acid (**7.5**).

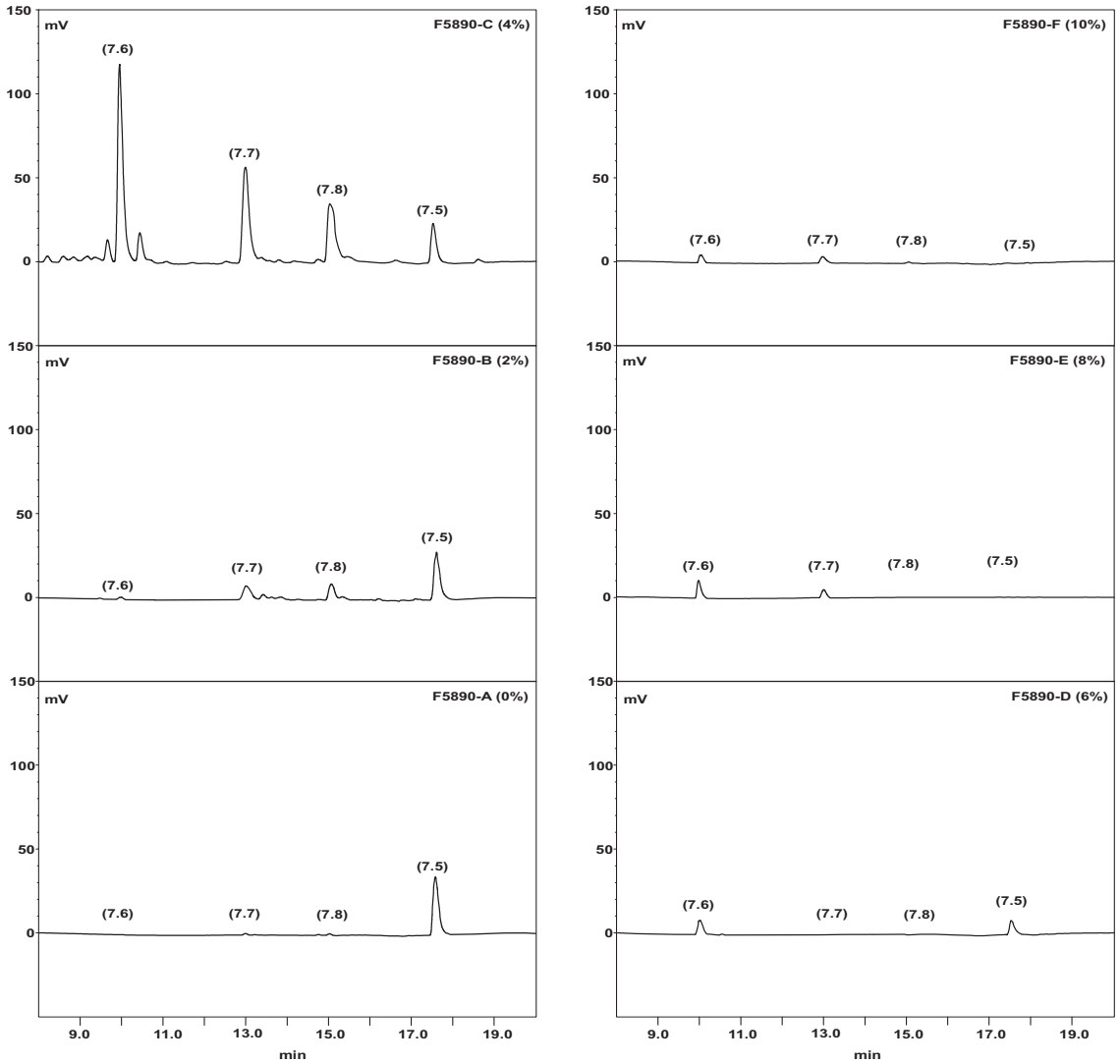


Figure 4.6: ELSD traces of α-cyclopiazonic acid (**7.5**), roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**) from extracts of *Penicillium* sp. (KK3T23) cultured on PYGA medium (pH 7.0±0.2; 25°C; 30 days) at salinity of 0, 2, 4, 6, 8 and 10%.

4.5 Discussion

The effect of salinity on the growth of these two marine-derived *Penicillium* isolates reflects the observations made by Munoz (2006) on two other *Penicillium* spp. isolated from a hypersaline environment. In that study, *P. chrysogenum* and *P. citrinum* showed that the NaCl concentrations for their growth ranged between 5 – 10%. An algal-derived *P. dravuni* (Janso *et al*., 2005) also showed greater growth with increasing salinity up to a level of 10% NaCl. A study on a sponge-derived isolate of *Penicillium* sp. showed that growth was not affected by seawater concentrations (Masuma *et al.,* 2001). The ability of marine-derived fungi to grow in saline conditions has been studied by using different concentrations of seawater by Barghoorn (1944), Sguros and Simms (1964) and Jones and Jennings (1964); NaCl and artificial sea water by Sguros and Simms (1964). In these studies, fungal growth was determined by dry weight measurements except for the study by Barghoorn (1944) who utilized colony diameter. These studies were reviewed by Jennings (1983) and summarized that the growth of higher marine fungi can be optimal at 100% seawater, but more frequently optimal growth is at a lower percentage. The results from this study showed that salinity affected the growth of the two *Penicillum* spp., however, a more extensive study needs to be done to obtain more comprehensive results because growth may also be dependent upon other parameters such as temperature whether or not adaptation by the fungi to salinity has occurred. The interacting effects of temperature and salinity on growth of marine fungi have been demonstrated by Ritchie (1957).

The effect of salinity on bioactivity of marine-derived *Penicillium* spp. has been described by Bugni and Ireland (2004). Three *Penicillium* spp. exhibited increasing antimicrobial activity with increasing concentrations of artificial sea water (Bugni and Ireland, 2004). A few studies have been reported on the effect of salinity on bioactivity of other marine-derived fungi. Three marine-derived *Aspergillus* spp. and an unidentified marine fungus showed enhanced antibacterial activity in the presence of seawater (Masuma *et al*., 2001). A marine fungus *Leptosphaeria oraemaris* (Miller and Savard, 1989) also showed that antibacterial activity was greatest at higher salinities (>22‰) and toxicity of extracts to brine shrimp larvae was greatest at 14, 18 and 22‰. These findings were consistent with the results obtained from *Penicillium* sp. (LY1L5) and *Penicillium* sp. (KK3T23) where the effect of salinity varied between different isolates.

Terverticillate *Penicillium* spp. are common producers of α-cyclopiazonic acid (**7.5**), together with other metabolites such as cyclopeptin (**7.7**) and viridicatin (**7.8**) (Frisvad *et al*., 2004). The metabolite profile of isolate KK3T23 correlates well with such terverticillate penicilli. Most α-cyclopiazonic acid-producers are terrestrial species such as *P. camemberti, P. commune, P. dipodomyicola, P. griseofulvum* and *P. palitans*. Only *P. palitans* is recorded as producing α-cyclopiazonic acid (**7.5**), cyclopeptin (**7.7**) and viridicatin (**7.8**) (Frisvad *et al*., 2004) and hence isolate KK3T23 may well be of this species especially as this isolate closely matches the morphological description of *P. palitans* as described by Pitt (1979). The marine-derived isolate KK3T23 produced similar metabolites to terrestrial isolates. This correlates well with the work of Sonjak *et al.* (2005) on *P. crustosum* strains isolated from various ecological niches. That study showed that metabolite production was consistent irrespective of geographic origin or habitat.

Cycloaspeptide A (**7.4**) was first isolated from *Aspergillus* sp. (Kobayashi *et al.*, 1987) and has only been found in five species of psychrotolerant *Penicillium*, namely *P. ribeum* (Dalsgaard *et al.*, 2004; Frisvad *et al*., 2006), *P. algidum* (Dalsgaard *et al*., 2005) and *P. jamensonlandense, P. lanosum* and *P. soppii* (Frisvad *et al*., 2006). It was also noted in this study that the ELSD traces of cycloaspeptide A (**7.4**) in isolate LY1L5 were more prominent than the cytotoxic α-cyclopiazonic acid (**7.5**) at all ranges of salinity (0 – 10%). The consistency in cycloaspeptide A (**7.4**) production by this isolate was similar to those reported for the cold-tolerant strains of *Penicillium*. Based on this, isolate LY1L5 is suggested to have a closer association with the groups of *Penicillium* that produced cycloaspeptide A (**7.4**).

Isolate LY1L5 has small, spinose conidia unlike the α-cyclopiazonic acid-producers that have bigger, smooth-walled conidia. Similarly isolate LY1L5 could not be matched with other cycloaspeptide A (**7.4**) producing *Penicillia*. Further work needs to be conducted to identify the species of isolate LY1L5.

Chapter 5

Secondary metabolites from marine-derived *Streptomyces* sp. (LA3L2)

5.1 Introduction

The genus *Streptomyces* is represented in nature by the largest number of species among all the genera of actinomycetes and numbers over 500 species. The name *Streptomyces* was introduced in 1943 for the aerial mycelium-producing actinomycetes. Members of this genus are Gram-positive filamentous microorganisms found predominantly in soil and in decaying vegetation. They are noted for their distinct earthy odour, resulting from production of a volatile metabolite, geosmin. Species of *Streptomyces* exhibit a unique morphological differentiation among the prokaryotes by the formation of spores on their aerial mycelium, superimposed upon the vegetative growth (Waksman, 1959).

5.1.1 Bioactive metabolites from marine-derived *Streptomyces* spp.

The interest in the antibiotics produced by *Streptomyces* is of long standing. It all began with the discovery of actinomycin by Waksman and his colleagues in 1940, followed by streptomycin in 1943 and numerous other compounds later. These included grisein and neomycin. During the last decade, there has been increasing number of novel metabolites possessing potent bioactivity isolated from marine-derived *Streptomyces* (Kelecom, 1999; Kelecom, 2002; Laatsch, 2006; Lam, 2006). Many of them are cytotoxic and come from a wide variety of chemical structures such as macrolides, α-pyrones, lactones, indoles, terpenes and quinones. Table 5.1 lists some selected novel bioactive metabolites that have been isolated from marine-derived *Streptomyces* from 1996 to 2007.

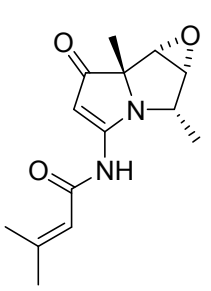
Table 5.1: Bioactive metabolites from marine-derived *Streptomyces* spp. reported during the period 1996 – 2007.

Isolates	Metabolites	Activities	References
Streptomyces sp. (marine sediment)	wailupamycin A 3-epi-5-deoxyenterocin	antibacterial	Sitachitta *et al*., 1996
Streptomyces sp. (marine sediment)	dihydrophencomycin methyl ester	antibacterial	Pusecker *et al*., 1997
S. halstedii olivaceous (marine sediment)	halawanones A and B	antibacterial	Ford *et al*., 1998
Streptomyces sp. (marine sediment)	cyclomarin A	antitumour; antiflammatory	Renner *et al*., 1999
Streptomyces sp. (seawater sample)	watasemycins A and B	antibacterial	Sasaki *et al*., 2002
S. hygroscopicus (marine fish, *Halichoeres bleekeri*)	halichoblelide	antitumour	Yamada *et al*., 2002
Streptomyces sp. (marine sediment)	komodoquinone A	neuritogenic activity	Itoh *et al*., 2003
Streptomyces sp. (unidentified marine invertebrate)	3,6-disubstituted indole 1 and 2	antitumour	Lopez *et al*., 2003
Streptomyces sp. (marine sediment)	bonactin	antibacterial; antifungal	Schumacher *et al*., 2003
S. aureoverticillatus (marine sediment)	aureoverticillactam	antitumour	Mitchell *et al*., 2004
Streptomyces sp.	caprolactones	antitumour	Stritzke *et al*., 2004
Streptomyces sp. (unidentified marine sponge)	dehydroxynocardamine desmethylenylnocardamine	weak inhibition against recombinant enzyme	Lee *et al*., 2005
Streptomyces sp.	chinikomycins A and B	antitumour	Li *et al*., 2005
Streptomyces sp. (marine sediment)	glyciapyrrole A	antitumour	Macherla *et al*., 2005
S. nodosus (marine sediment)	lajollamycin	antibacterial; antitumour	Manam *et al*., 2005
Streptomyces sp. (marine sediment)	daryamides A, B and C	antitumour	Asolkar *et al*., 2006
S. corchorusii (marine sediment)	resistomycin tetracenomycin D	antitumour, weak antibacterial	Adinarayana *et al*., 2006
Streptomyces sp. (marine sediment)	azamerone	antitumour	Cho *et al*., 2006
Streptomyces sp. (marine plant, *Salicornia herbacea*)	methylanthraquinone	antitumour	Huang *et al*., 2006
Streptomyces sp. (marine sediment)	propylanthraquinone	antibacterial	Poumale *et al*., 2006
Streptomyces sp. (marine sediment)	piperazimycins A, B and C	antitumour	Miller *et al*., 2007

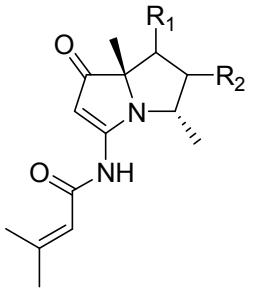
5.1.2 Pyrrolizidines

Pyrrolizidine alkaloids are a group of compounds that contain nitrogen in a heterocyclic ring. Although these metabolites are commonly found in plants (Culvenor, 1980), a novel member of the pyrrolizidines was discovered from an *Actinosporangium* sp. in 1980 (Doyle *et al*., 1980; Nettleton *et al*., 1980). The compound, named bohemamine (**5.1**), was found to have an unusual tricyclic structure reminiscent of the pyrrolizidine alkaloids, except for the inconsistency in the substitution pattern.

In recent years, it has been established that *Streptomyces* spp. are the main producers of the bohemamines. A new compound, NP25302 (**5.5**), was isolated from *Streptomyces* sp. in 2003 (Zhang *et al*., 2003b), followed by three others, namely bohemamine B (**5.2**), bohemamine C (**5.3**) and 5-chlorobohemamine C (**5.4**) which were isolated from a marine-derived *Streptomyces* sp. (Bugni *et al*., 2006).

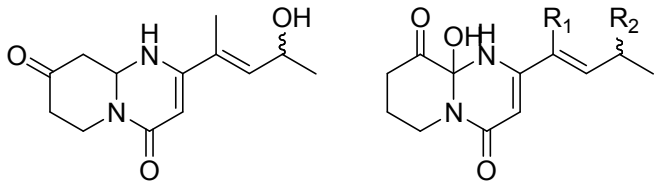


5.1



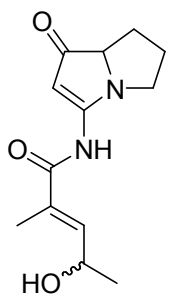
5.2 R_1 = H; R_2 = ····OH
5.3 R_1 = ····OH ; R_2 = H
5.4 R_1 = ····OH; R_2 = ◄Cl
5.5 R_1 = H; R_2 = H

A revision of the structure of three bicyclic alkaloids (**5.6**, **5.7** and **5.8**) that had been isolated from a *Streptomyces* sp. (Hu *et al*., 2003) led to three new metabolites (**5.9**, **5.10** and **5.11**) being described that possessed structures similar to bohemamines (Snider *et al*., 2004).

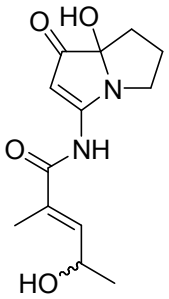


5.6

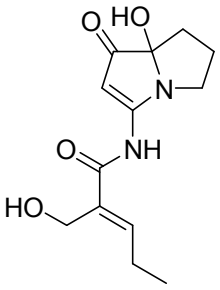
5.7 R_1=Me; R_2 = OH
5.8 R_1=CH_2OH; R_2 = H



5.9



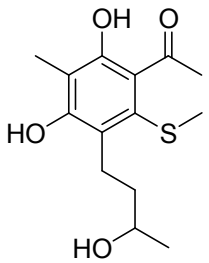
5.10



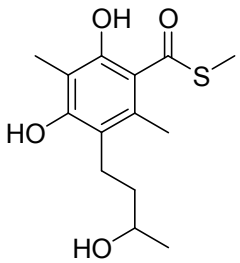
5.11

5.1.3 *S*-methyl benzothioates

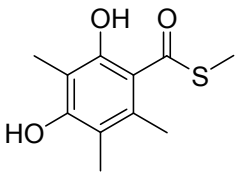
A novel antitumor antibiotic, resorthiomycin was produced in a broth culture of *S. collinus* and the unusual structure (**5.12**) was proposed (Tahara *et al*., 1990). The structure was revised in 1991 (Tahara *et al*., 1991) and a rare *S*-methyl benzothioate structure (**5.13**) was proposed in the revision. *S. collinus* remained the only reported producer of this unusual structural type until a comparable structure (**5.14**) was identified from a sclerotium-colonizing isolate of the fungus, *Mortierella vinacea* (Soman *et al*., 1999). To date, resorthiomycin (**5.13**) and mortivinacin A (**5.14**) are the only secondary metabolites reported for the *S*-methyl benzothioate group of metabolites.



5.12



5.13



5.14

5.1.4 *Streptomyces* sp. (LA3L2)

Streptomyces sp. (LA3L2) was obtained from liquid portions of a New Zealand marine tunicate, collected at Lyttleton Harbour in May 2004. Extracts were obtained from cultures of isolate LA3L2 grown on SCA and SCB media and coded F5958 and F5936, respectively. Both extracts were assayed for cytotoxic and antimicrobial activities. F5958 exhibited significant cytotoxicity (IC_{50}784 ng/mL) against P388 cells, but was not active against *B. subtilis*, *P. aeruginosa* and *C. albicans*. F5936 was not active against P388 cells or the microorganisms tested (see **Chapter Three** – *Preliminary screening*).

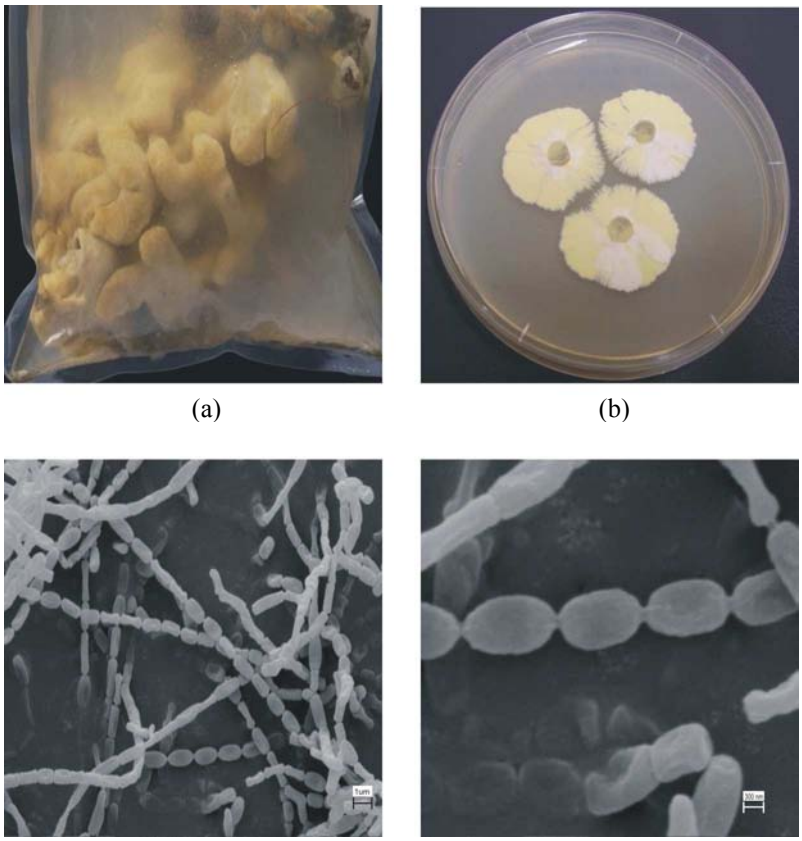
This isolate was further investigated because it showed good activity against P388 cells (IC_{50}784 ng/mL), but the active metabolites were not significantly produced. For this reason, a large scale cultivation of *Streptomyces* sp. (LA3L2) was carried out to generate the production of metabolites. The ability of the CapNMR technique to chemical characterization the desired metabolites using the one and two dimensional NMR techniques is fully investigated.

Streptomyces sp. (LA3L2) was obtained from the liquid portions of a New Zealand marine tunicate, collected at Lyttleton Harbour in May 2004. Extracts were obtained from cultures of isolate LA3L2 grown on SCA and SCB media and coded F5958 and F5936, respectively. Both extracts were assayed for cytotoxic and antimicrobial activities. F5958 exhibited significant cytotoxicity (IC_{50}784 ng/mL) against P388 cells, but was not active against *B. subtilis*, *P. aeruginosa* and *C. albicans*. F5936 was not active against P388 cells or the microorganisms tested (see **Chapter Three** – *Preliminary screening)*.

5.2 Characterization of isolate LA3L2

The isolate appeared as a yellowish colony on SCA medium (see Figure 5.1 (a-b)). The isolate was initially identified as *Streptomyces* sp. based on the characteristic 'earthy odour' of the culture and on its cultural characteristics. The growth of hyphae was initially whitish yellow and then changed to yellow when mature, with the formation of white aerial mycelia. The substrate mycelium of this isolate adhered strongly to the medium, while the aerial mycelium was thicker than the substrate mycelium. This isolate was Gram-positive and grew best in between 20 - 36°C, again an important characteristic of the genus *Streptomyces*.

The identification of isolate LA3L2 as belonging to the genus *Streptomyces* was further supported by SEM microscopic characteristics. Preobrazhenskaia *et al*. (1959) stated that cultures of *Streptomyces* with a white, yellow, greenish yellow, yellow-gray, pink or lilac mycelium have smooth spores. This isolate produced a chain of single celled smooth surfaced spores (see Figure 5.1 (c-d)). Details of the procedures for isolation, cultivation and identification are given in **Chapter Two** (*Experimental*).



(a) (b)

(c) (d)

Figure 5.1 Characterization of a marine-derived *Streptomyces* sp. (LA3L2); (a) New Zealand tunicate; (b) colony on SCA (30 days); (c) chain of single celled spores under SEM and (d) smooth-surfaced spores under SEM.

Part A: Pyrrolizidines from F5958

5.3 Investigation of a small-scale extract (F5958)

An aliquot of the crude extract (F5958) was chromatographed on reverse-phase C18 HPLC using the standard gradient (see **Chapter Two** - *Experimental*) revealing three main compounds, NAM 5-1, NAM 5-2 and NAM 5-3 appearing at R_t 10.0 – 12.5 min. The extract was further assayed to determine the compound(s) responsible for the cytotoxicity. The result from the HPLC MTT plate assay (see **Chapter Two** - *Experimental*) indicated that activity was not due to any of the three main compounds (NAM 5-1, NAM 5-2 and NAM 5-3), but correlated with the compounds eluted over the period R_t 15.0 – 18.5 min (see Figure 5.2).

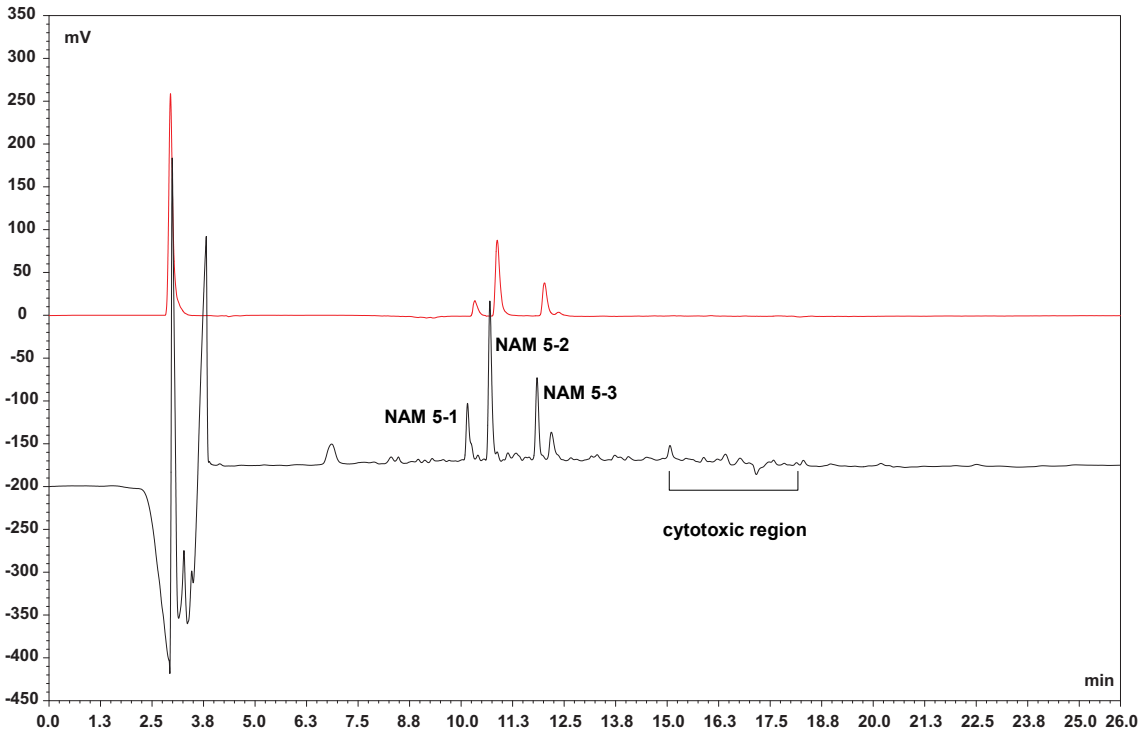


Figure 5.2: HPLC chromatogram of F5958 showing overlay of ELSD detection (top) for compounds NAM 5-1, NAM 5-2 and NAM 5-3.

Compounds NAM 5-1, NAM 5-2 and NAM 5-3 were shown to have virtually identical UV chromophores, suggesting that they were very closely related in structure (Figure 5.3).

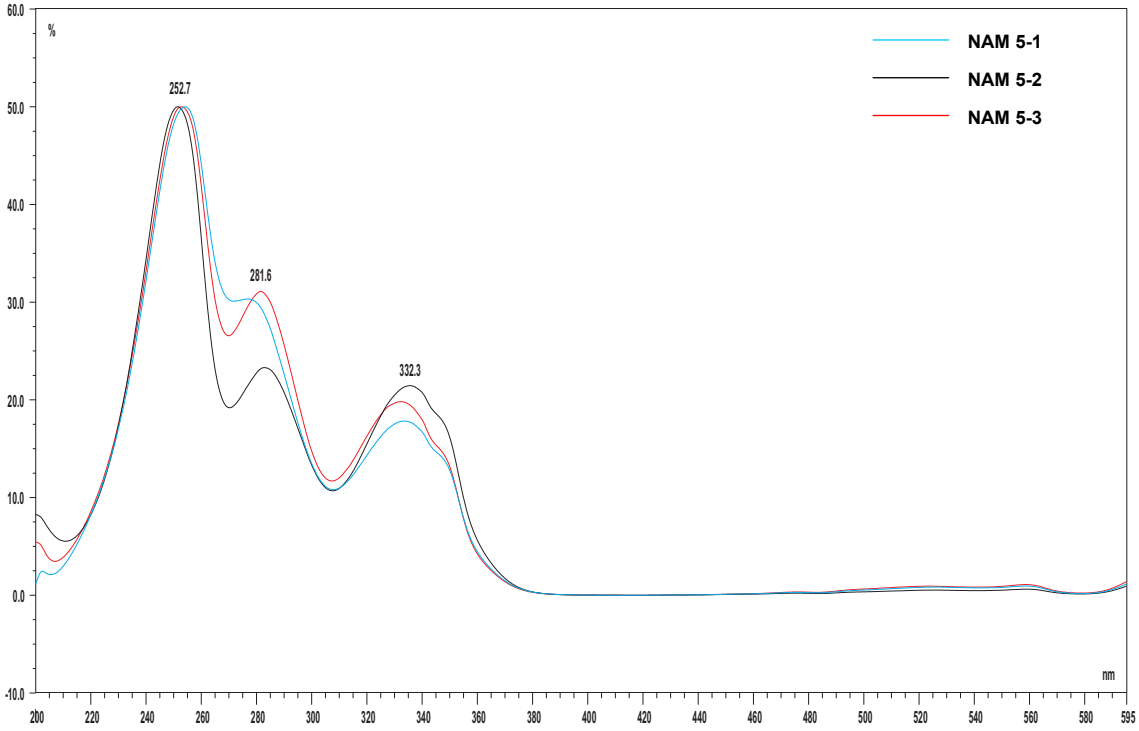


Figure 5.3: UV profile overlay for compounds NAM 5-1, NAM 5-2 and NAM 5-3.

Although compounds NAM 5-1, NAM 5-2 and NAM 5-3 were eluted over the inactive region, further investigation was carried out to establish the nature of the metabolites. The HPLC fractionation of crude extract of F5958 successfully purified compounds NAM 5-2 and NAM 5-3, however no suitable amount of compound NAM 5-1 was obtained in the attempt. Compounds NAM 5-2 and NAM 5-3 were investigated using the CapNMR technique discussed in **Sections 5.3.1** and **5.3.2** below. Further attempt to investigate the metabolites eluted over the cytotoxic region (R_t 15.0 – 18.5 min) is discussed in **Section 5.4**.

5.3.1 Compound NAM 5-2

The ^{1}H NMR spectral data for compound NAM 5-2 obtained with the CapNMR probe in CD_3OD clearly showed the presence of three singlet methyls at δ_H 1.37, 1.96 and 2.22 and a doublet methyl at δ_H 1.47 (Figure 5.4).

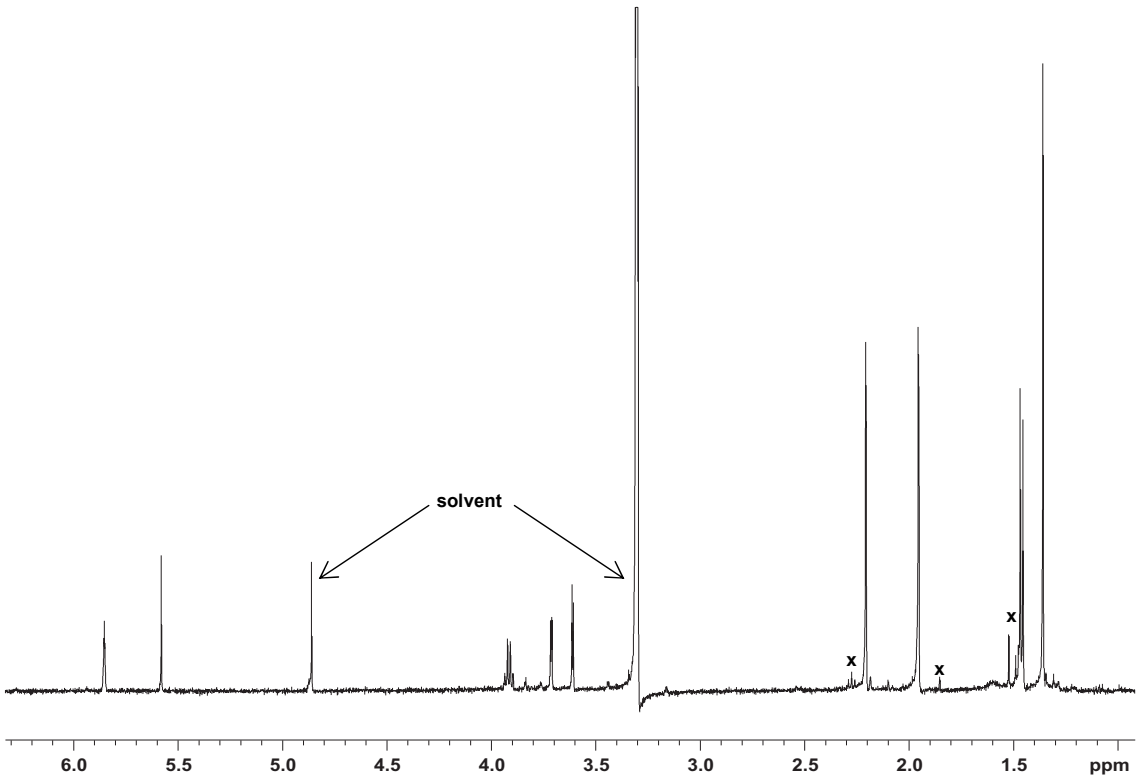


Figure 5.4: ^{1}H NMR spectrum of compound NAM 5-2 in CD_3OD obtained from F5958. Crosses indicate impurities.

This compound was shown to have a molecular mass of 263 Da $([M+H])^+$ (Figure 5.5). A search in the AntiMarin database for compounds with four methyl groups (three singlets and one doublet) and molecular weight of 261 – 263 Da, revealed nine possible structures for compound NAM 5-2.

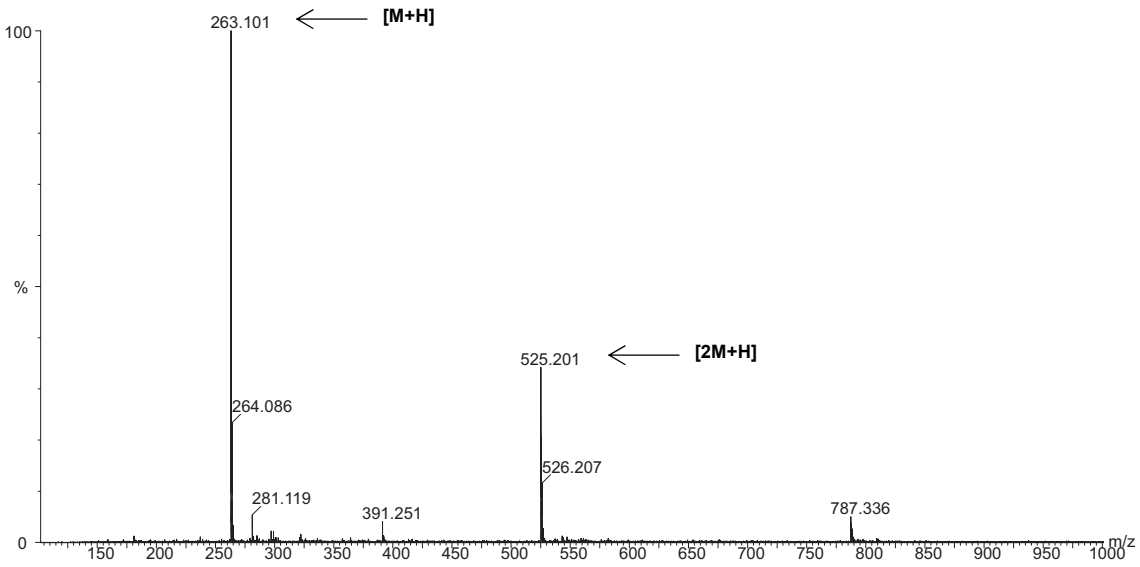
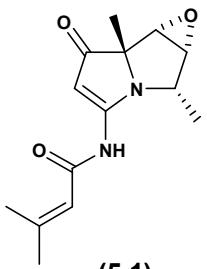


Figure 5.5: ESIMS spectrum of compound NAM 5-2.

Eight of the known compounds were excluded due to the presence of methylene protons ($-CH_2-$) in their structures, which were not detected in the ^1H NMR spectrum presented in Figure 5.4. Hence, bohemamine (**5.1**) was the only potential structure for compound NAM 5-2.



(5.1)

It should be noted that the structure of bohemamine (**5.1**), first reported in 1980 (Doyle *et al.,* 1980) was later renumbered when Bugni and co-workers (2006) isolated the known bohemamine (**5.1**). The numbering of bohemamine (**5.1**) is presented in Figure 5.6.

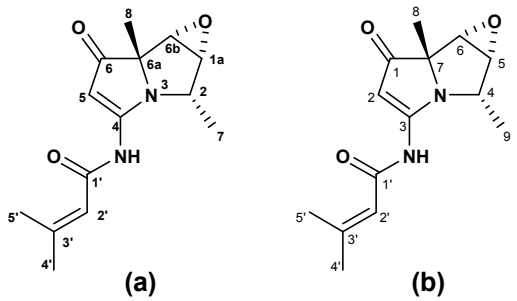


Figure 5.6: Numbering of bohemamine (**5.1**): (a) from Doyle *et al*. (1980); (b) from Bugni *et al*. (2006).

The proton chemical shifts of compound NAM 5-2 were consistent with bohemamine (**5.1**) based on the comparison of its ^{1}H NMR data (Table 5.2) to those reported for bohemamine (**5.1**) in $CDCl_3$ (Doyle *et al*., 1980) and in DMSO-d_6 (Bugni *et al*., 2006). The structure of compound NAM 5-2, was thus assigned as bohemamine (**5.1**).

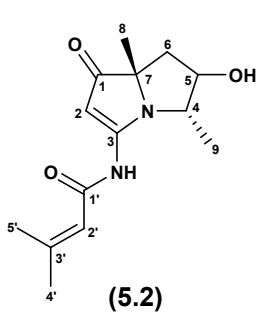
Table 5.2: Comparison of ^{1}H NMR data of compounds NAM 5-2 and (**5.1**).

Position [a]	δ ^{1}H, ppm multiplicity (J_{HH} Hz)		
	NAM 5-2[b]	5.1[c]	5.1[d]
1			
2	5.58, s	5.67, s	5.34, s
3			
4	3.89, q (6.6)	3.87, q (~7)	3.80, br, q (6.7)
5	3.61, d (3.0)	3.58, q	3.73, br, d (3.4)
	3.71, d (3.0)		
6	3.72, d (3.0)		3.60, d (3.4)
7			
8	1.37, s	1.38, s	1.20, s
9	1.47, d (6.8)	1.57, d (~7)	1.35, d (6.7)
1'			
2'	5.85, s	5.88, br, s	5.90, dq (1.3, 1.0)
3'			
4'	1.96, s	1.94, d (~1)	1.87, d (1.0)
5'	2.22, s	2.28, d (~1)	2.12, d (1.3)
NH		9.2, br, s	10.10, br, s

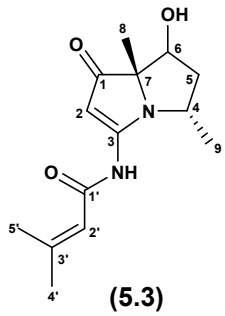
[a] These positions were renumbered (Bugni *et al*., 2006); [b] These data were recorded at 500 MHz using CD_3OD; [c] These data were recorded using $CDCl_3$ (Doyle *et al*., 1980); [d] These data were recorded using DMSO-d_6 (Bugni *et al*., 2006).

5.3.2 Compound NAM 5-3

Based on the similarity of their UV chromophores, NAM 5-3 was closely related in structure to compound NAM 5-2 (see Figure 5.3). The structural feature of compound NAM 5-2 (**5.1**) was then used as a basis to identify compound NAM 5-3. Compound NAM 5-3 had a molecular mass of 265 Da $([M+H])^{+}$. A search in the AntiMarin database using the molecular weight of 263 – 265 Da led to two known bohemamines, namely bohemamine B (**5.2)** and bohemamine C (**5.3**).



(5.2)



(5.3)

Both compounds (**5.2** and **5.3**) were reported as having similar UV spectral data to compound NAM 5-3, therefore the investigation using the ^{1}H NMR data was necessary. The ^{1}H NMR spectrum of compound NAM 5-3 (Figure 5.7) indicated almost identical methyl signals at δ 1.31, 1.95 and 2.21, and almost similar signals of vinyl protons at δ 5.77 and 5.94, to those observed in Figure 5.4 for compound NAM 5-2. There were dissimilarities in the chemical shifts for a doublet methyl (δ 1.07) and methine protons (δ 4.0 and 4.6) for compound NAM 5-3 compared to those of compound NAM 5-2. These variations were likely due to the presence of two additional hydrogen atoms in compound NAM 5-3 in comparison to compound NAM 5-2.

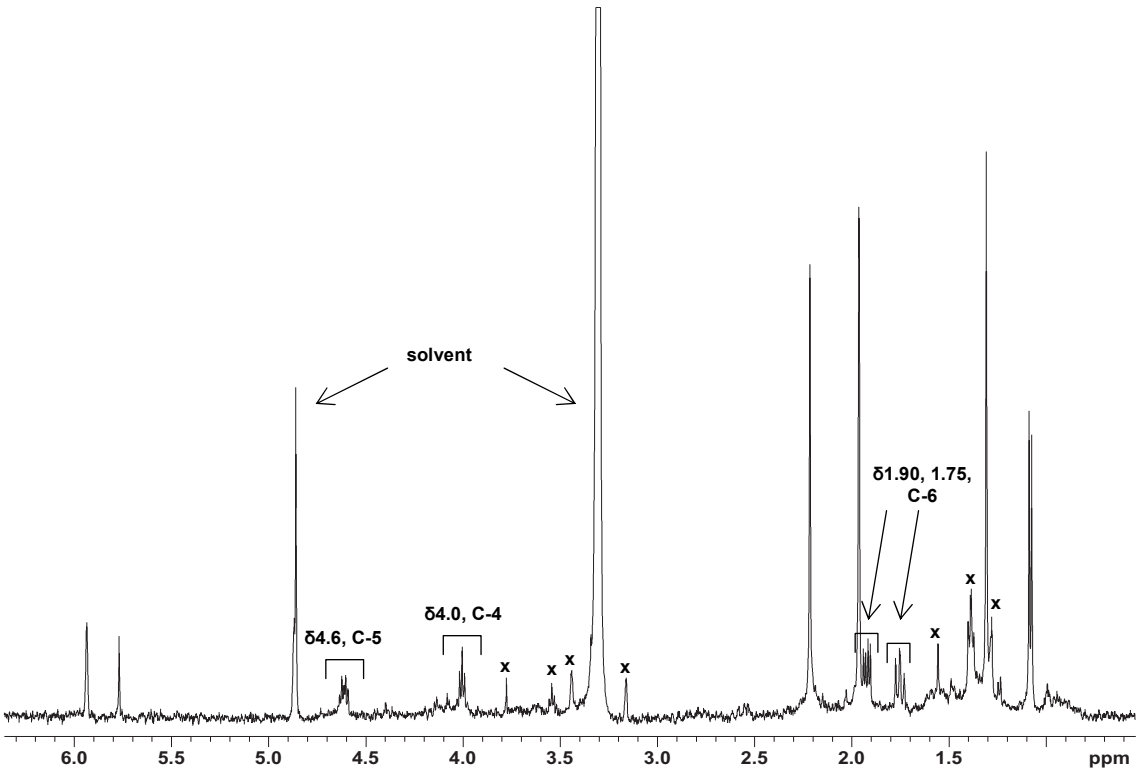


Figure 5.7: ^{1}H NMR spectrum of compound NAM 5-3 in CD_3OD obtained from F5958. Crosses indicate impurities.

The ^{1}H NMR data of compound NAM 5-3 were compared to those reported for bohemamines B (**5.2**) and C (**5.3**) (Bugni *et al*., 2006). The results are shown in Table 5.3. It was deduced from Table 5.3 that the data of compound NAM 5-3 were more comparable to bohemamine B (**5.2**) than to those reported for bohemamine C (**5.3**). Variations in the values were recognized, however this was expected since the chemical shifts of compound NAM 5-3 were referenced against a different solvent peak to those of bohemamine B (**5.2**). Based on these data, compound NAM 5-3 was identified as bohemamine B (**5.2**).

Table 5.3: Comparison of ^{1}H NMR data of compounds NAM 5-3 to those reported for compounds **5.2** and **5.3**.

Position	δ^{1}H, ppm multiplicity (J_{HH} Hz)		
	NAM 5-3[a]	5.2[b]	5.3[b]
1			
2	5.77, s	5.56, s	5.40, s
3			
4	4.0, t (6.5, 6.4)	3.86, dq (6.5, 6.4)	3.92, dq (6.7, 8.7)
5	4.6, dddd (10.0, 5.6, 6.4, 3.8)	4.44 dddd (10.1, 5.9, 6.4,	α 1.75, d (13.2)
6	1.75, dd (11.9, 10.0)	α 1.55, dd (12.2, 10.1)	3.84, t (3.9)
	1.90, dd (11.9, 5.6)	β 1.69, dd (12.2, 5.9)	
7			
8	1.31, s	1.13, s	1.07, s
9	1.07, d (6.6)	0.90, d (6.5)	1.25, d (6.7)
1'			
2'	5.94, br, s	5.98, dq (1.0, 0.9)	5.98, br, s
3'			
4'	1.95, s	1.88, d (0.9)	1.88, br, s
5'	2.21, s	2.12, d (1.0)	2.12, br, s
NH	-	10.24, br, s	10.24, br, s
OH	-	4.82, d (3.5)	4.92, d (3.5)

[a] These data were recorded at 500 MHz in CD_3OD; [b] These data were recorded at 500 MHz in DMSO-d_6 (Bugni *et al*., 2006).

Part B: A new *S*-methyl benzothioate from F5958-A

5.4 Investigation of a large scale extract (F5958-A)

As the small scale extract (F5958) produced only small amount of metabolites over the cytotoxic region (R_t 15.0 – 18.5 min), the isolate was re-grown on a large scale to generate the production of the desired metabolites. Extraction of 60 plates yielded a dark brown crude extract (45.7 mg) coded F5958-A. The bioactivity of F5958-A was compared to that of the small scale extract (F5958). P388 assay showed that F5958-A possessed greater cytotoxicity (IC_{50}383 ng/mL) than F5958 (IC_{50}784 ng/mL). An aliquot of F5958-A was screened by HPLC and the chromatogram showed that it contained three main compounds; namely NAM 5-1, NAM 5-2 and NAM 5-4 (see Figure 5.8).

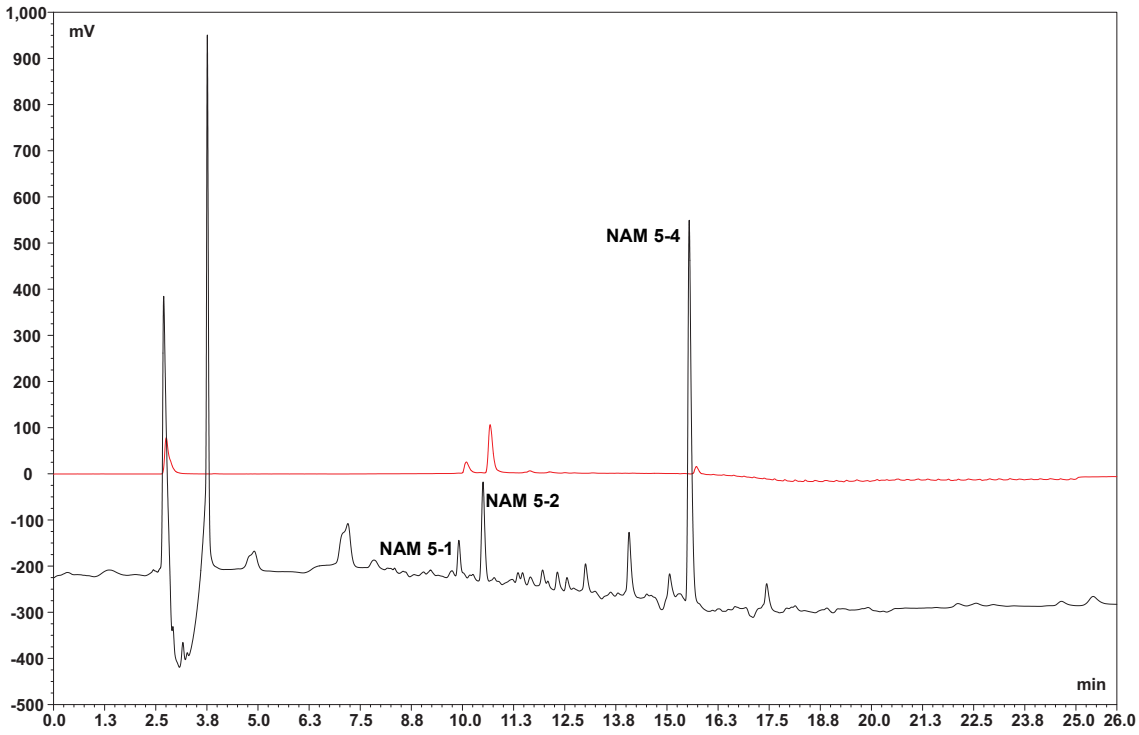


Figure 5.8: HPLC chromatograms of F5958-A showing overlay of ELSD detection (top) for compounds NAM 5-1, NAM 5-2 and NAM 5-4.

From these three peaks, only two (NAM 5-1 and NAM 5-2) were in common with those for F5958 based on the UV spectra. Compound NAM 5-3 was no longer present in this F5958-A extract. Peak NAM 5-4 was an additional compound eluted at 15.5 min to those produced by F5958. The UV spectra of compounds NAM 5-1, NAM 5-2 and NAM 5-4 are shown in Figure 5.9.

It was interesting to note that the HPLC MTT plate assay of F5958 showed that the cytotoxic region was detected over the region 15.0 – 18.5 min. As there was a practicable amount of compound NAM 5-4 produced in the cytotoxic region, an attempt was made to isolate and identify this compound and establish if this was a new metabolite.

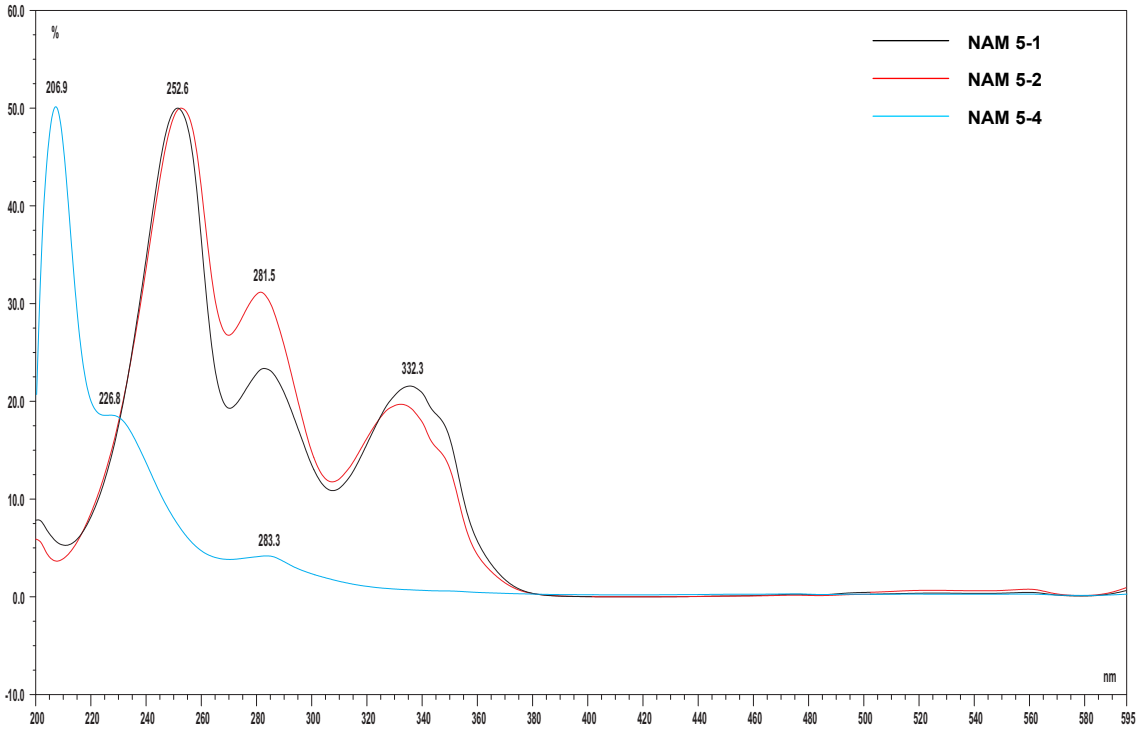


Figure 5.9: UV profile overlay for compounds NAM 5-1, NAM 5-2 and NAM 5-4 from F5958-A.

5.4.1 Isolation and purification of compound NAM 5-4

F5958-A was fractionated to isolate the three compounds NAM 5-1, NAM 5-2 and NAM 5-4. The extract was initially defatted with petroleum ether (Pet. Ether) yielding 21.6 mg of Pet. Ether layer (Fraction 1) and further partitioning with H_2O and EtOAc (1:1) resulted in 1.4 mg H_2O layer (Fraction 2) and 22.7 mg EtOAc layer (Fraction 3). Fraction 3 was further chromatographed on HPLC to obtain pure compounds NAM 5-1 and NAM 5-2 and a sub-fraction 3a (see Figure 5.10).

An aliquot of sub-fraction 3a was injected on to the HPLC and the fractions were collected into a microtitre plate (Figure 5.11). The pure compound NAM 5-4 was obtained from well F10 of the microtitre plate and analyzed using the CapNMR technique. Details of the experimental are shown in **Chapter Two** (*Experimental*). Further work to elucidate the structure of compound NAM 5-4 is discussed in the following sections. No further work was carried out on compound NAM 5-1 due to insufficient ^{1}H NMR spectral data.

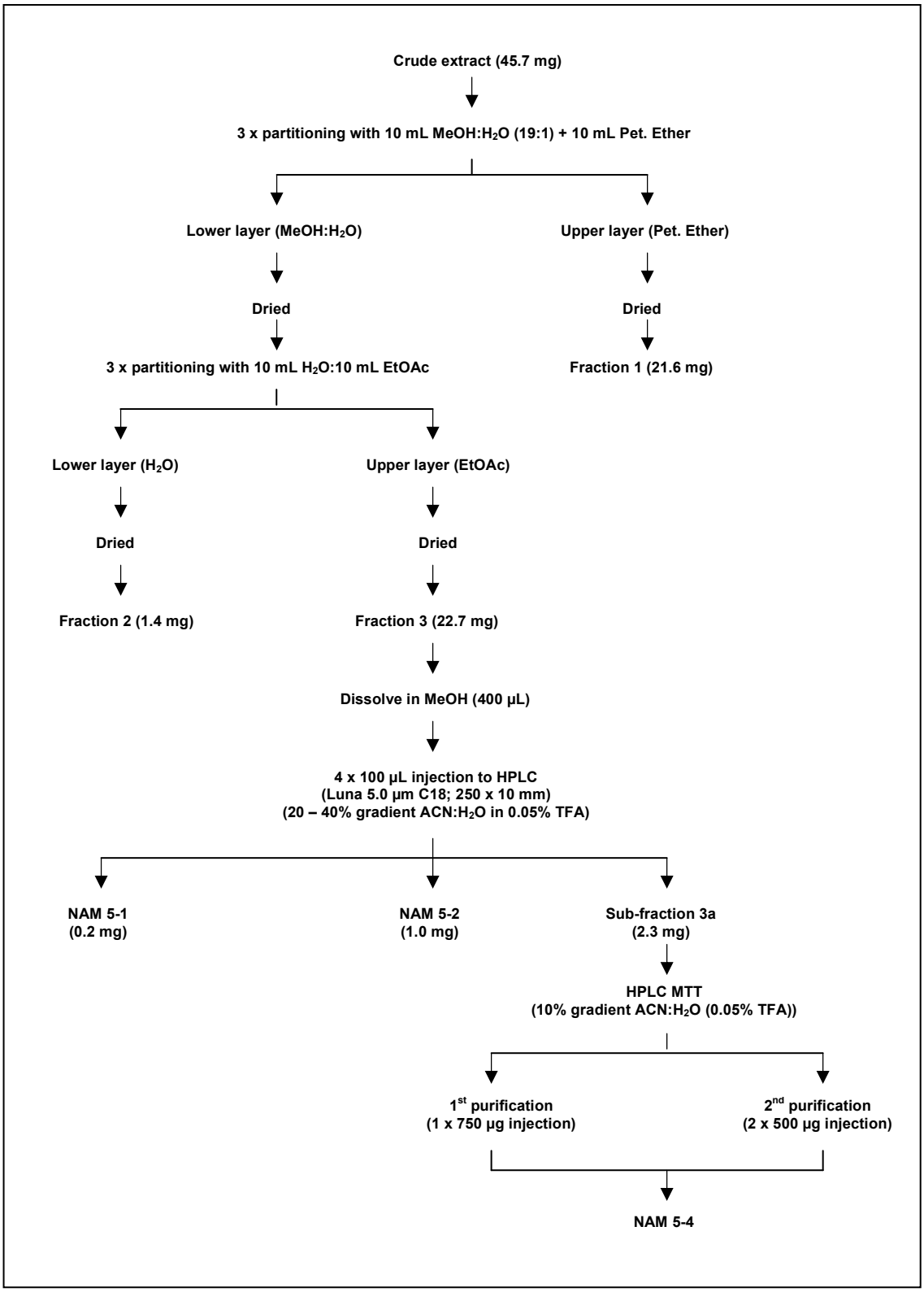


Figure 5.10: Isolation and purification of compound NAM 5-4 from F5958-A.

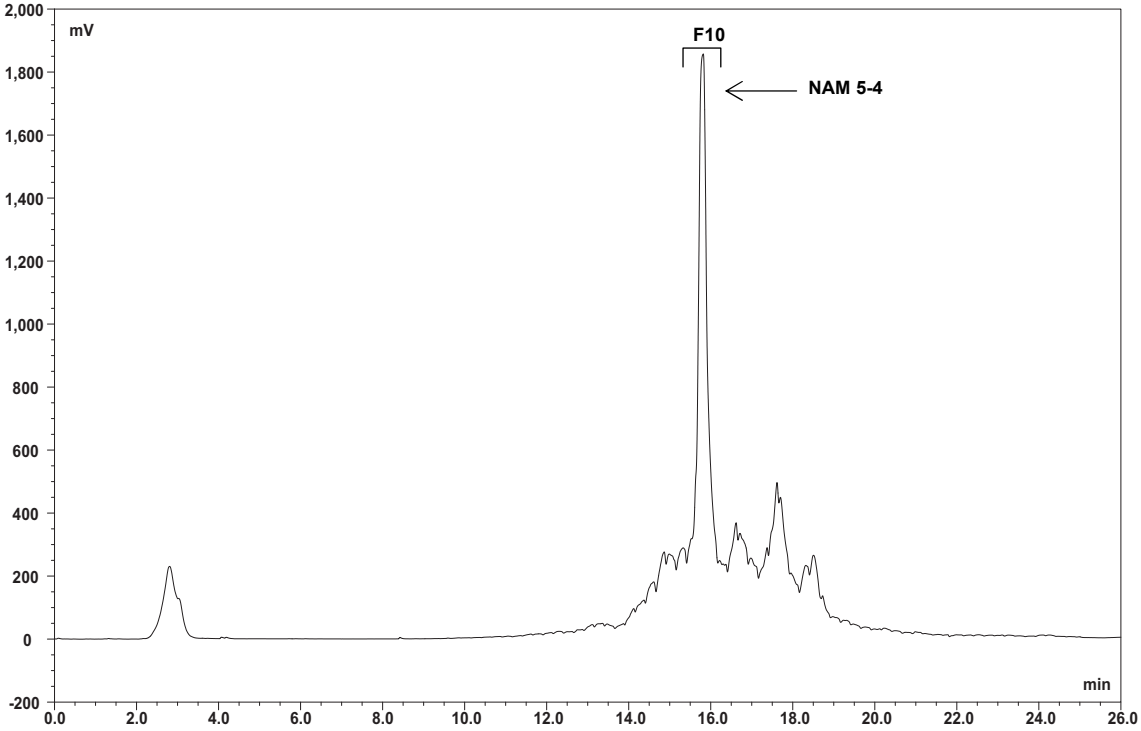


Figure 5.11: HPLC chromatogram of F5958-A (sub-fraction 3a) showing the main compound NAM 5-4 collected into well F10 of the microtitre plate (from 1 x 750 µg injection).

5.4.2 Structural elucidation of NAM 5-4

Compound NAM 5-4 was obtained from HPLC fractionation of sub-fraction 3a. The first attempt at purification yielded an almost pure compound (4 µg) as the ^{1}H NMR spectrum displayed sharp and well defined signals in (Figure 5.12). The spectrum indicated the presence of three singlet methyls at δ_H 2.07, 2.18 and 2.41 and an isopropyl group, represented by two doublets at δ_H 1.29 and to a multiplet methine group at δ_H 3.1.

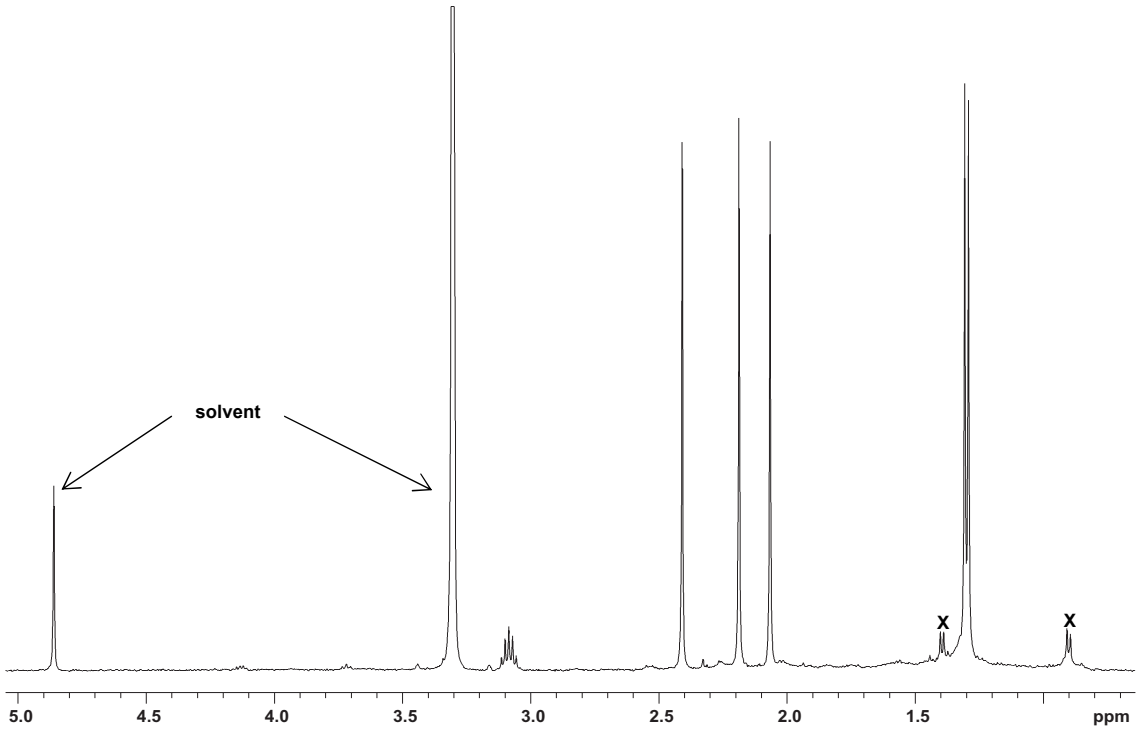


Figure 5.12: ^{1}H NMR spectrum of NAM 5-4 in CD_3OD obtained from first HPLC MTT purification. Crosses indicate impurities.

ESIMS data for this compound (see Figure 5.13) indicated that the mass to be 255 Da ($[M+H]^+$). A search in AntiMarin database using the spectral data from ^1H NMR and ESIMS resulted in no hits for any compounds within the database.

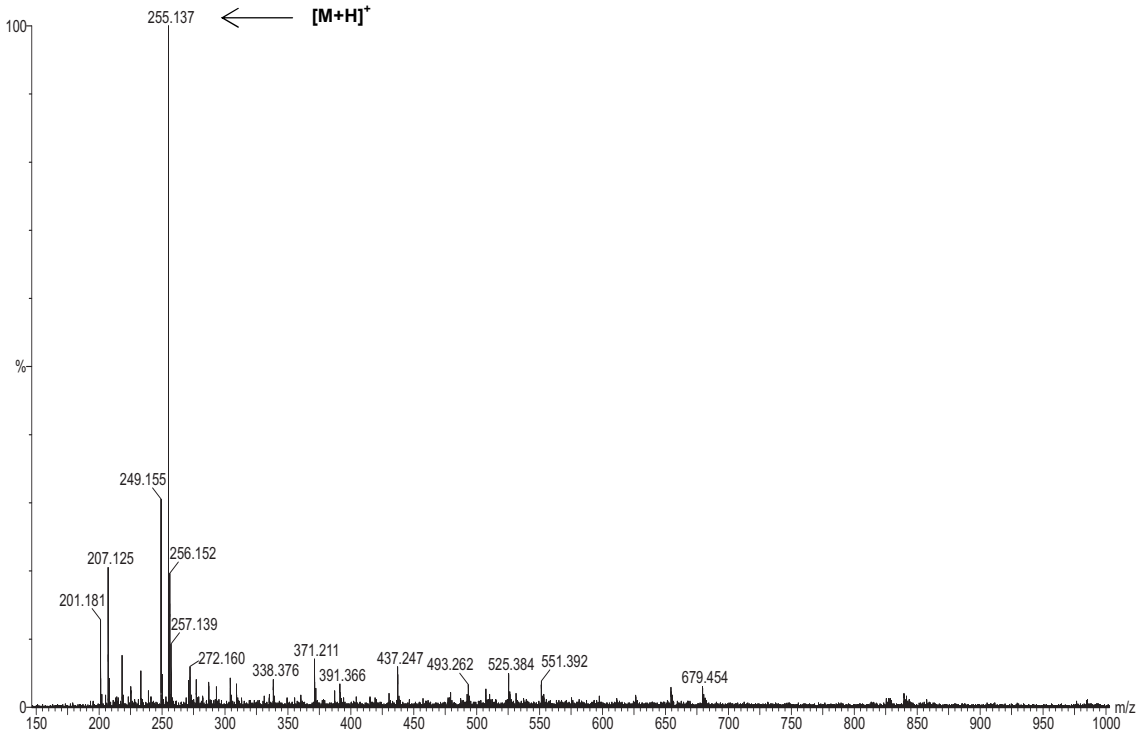


Figure 5.13: ESIMS spectrum of compound NAM 5-4.

As the amount of pure compound obtained was relatively low for further analysis, more of sub-fraction 3a was purified to collect more material. Sub-fraction 3a (2 x 500 µg) was injected into the HPLC to collect approximately 20 µg of pure material using the microtitre plate. Even though some minor impurities were still present (as illustrated in the ^{1}H NMR spectrum (Figure 5.14)), HMBC and HSQC DEPT experiments were performed and the results were sufficient to elucidate the structure of NAM 5-4.

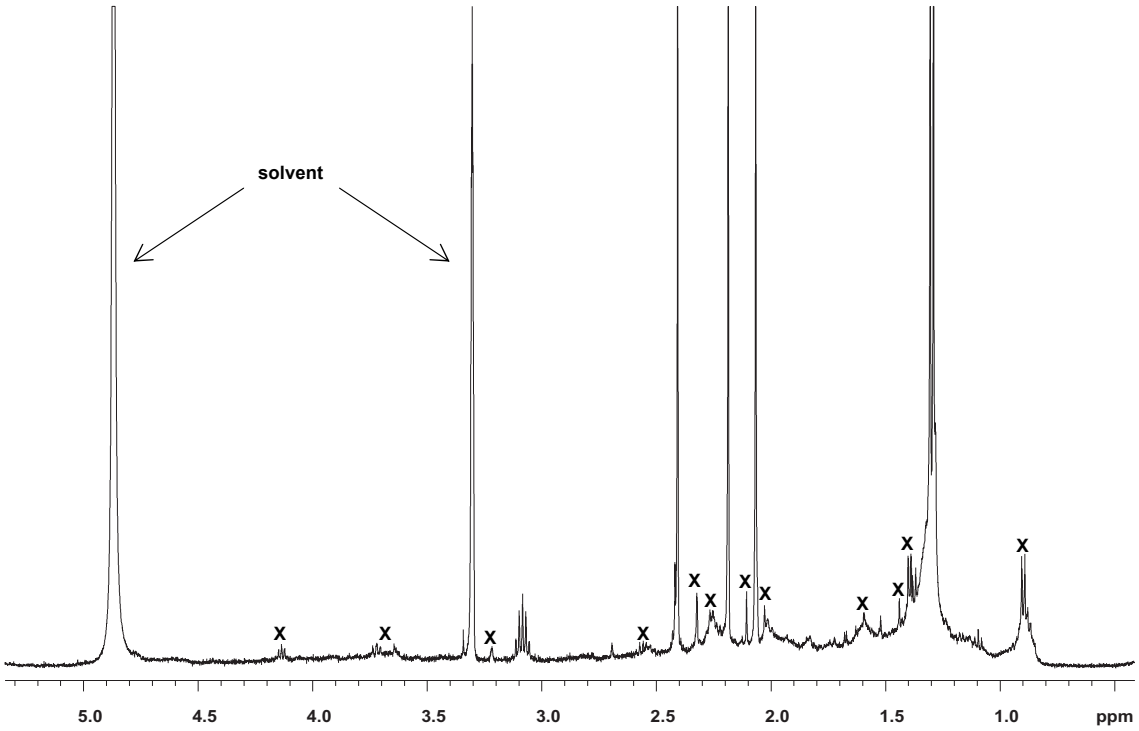


Figure 5.14: ^{1}H NMR spectrum of compound NAM 5-4 in CD_3OD obtained from second HPLC MTT purification. Crosses indicate impurities.

From the HSQC DEPT spectrum (Figure 5.15), the chemical shifts of the protons at δ 1.29, 2.07, 2.18 and 2.41 were correlated with the chemical shifts of their directly bonded carbons ($^1J_{CH}$ couplings).

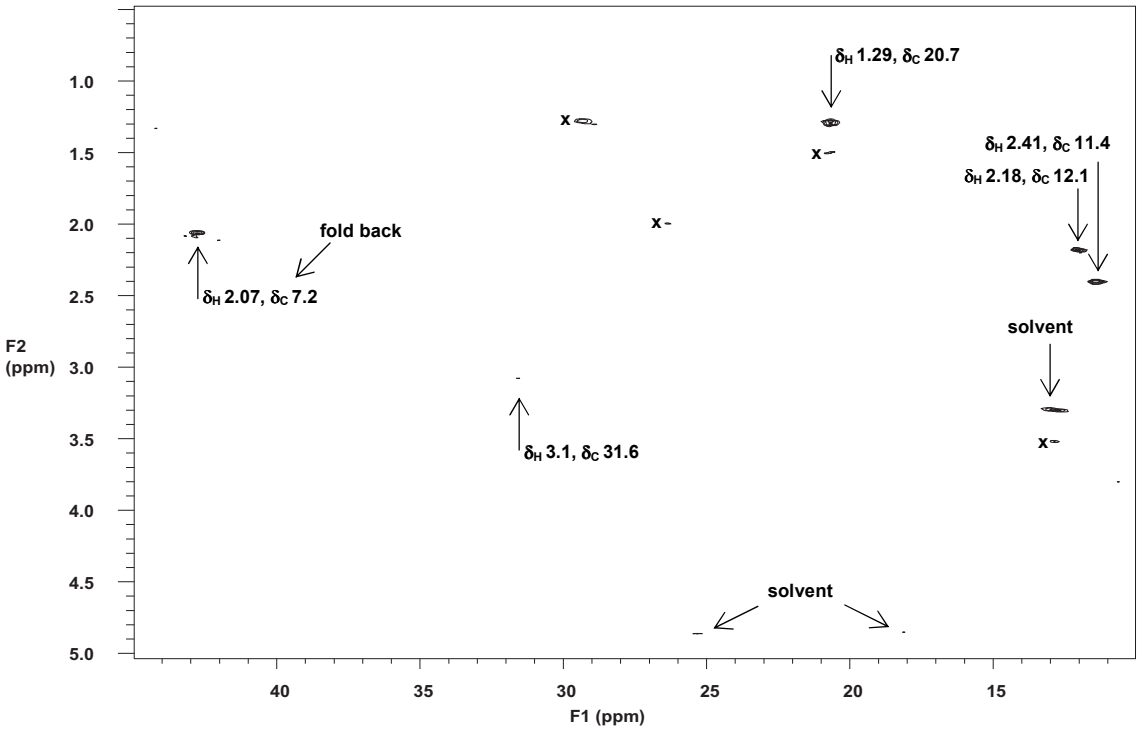


Figure 5.15: HSQC DEPT experiment of compound NAM 5-4. Crosses indicate impurities.

The HMBC spectrum clearly illustrated the presence of a hexasubstituted benzenoid system (Figure 5.16). The position of one aryl methyl group at δ 2.07 was established by strong HMBC correlations with two oxygen-bearing carbons (C-2, δ 149.2 and C-4, δ 155.5) and with one higher field carbon (C-3, δ 110.2), thus placing this group between two oxygenated aromatic carbons. The position of the other aryl methyl group was further established by strong HMBC connections of the signal at δ 2.18 with one oxygen-bearing carbon (C-4, δ 155.5) and two carbons (C5, δ 115.5 and C-6, δ 139.9). One of the two remaining aromatic carbons was substituted by an isopropyl group, proved by a long-range correlation of two methyl groups (δ 1.29) with the carbon (C-6, δ 139.9), leaving the C-1 position to be substituted by the carbonyl group (C-7, δ 198.4), which had a long-range HMBC correlation to the methyl group (δ 2.41). The long range couplings of this compound are shown schematically in Figure 5.17.

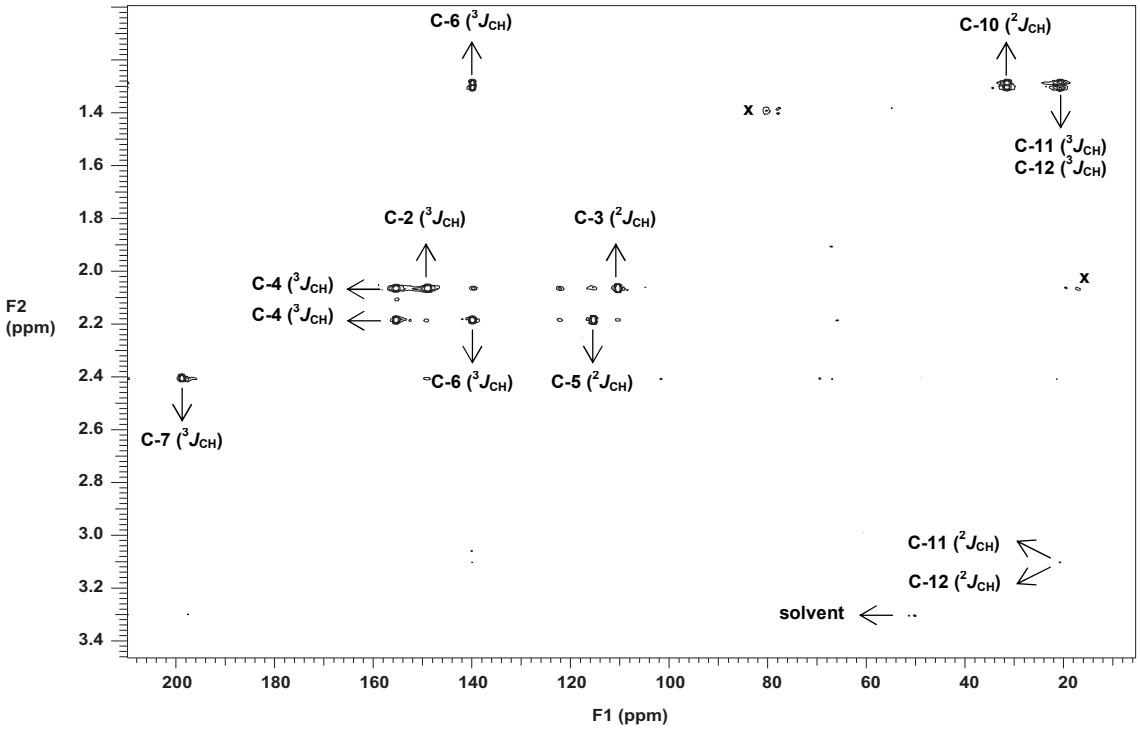


Figure 5.16: HMBC spectrum of compound NAM 5-4. Crosses indicate impurities.

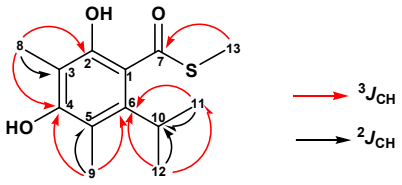
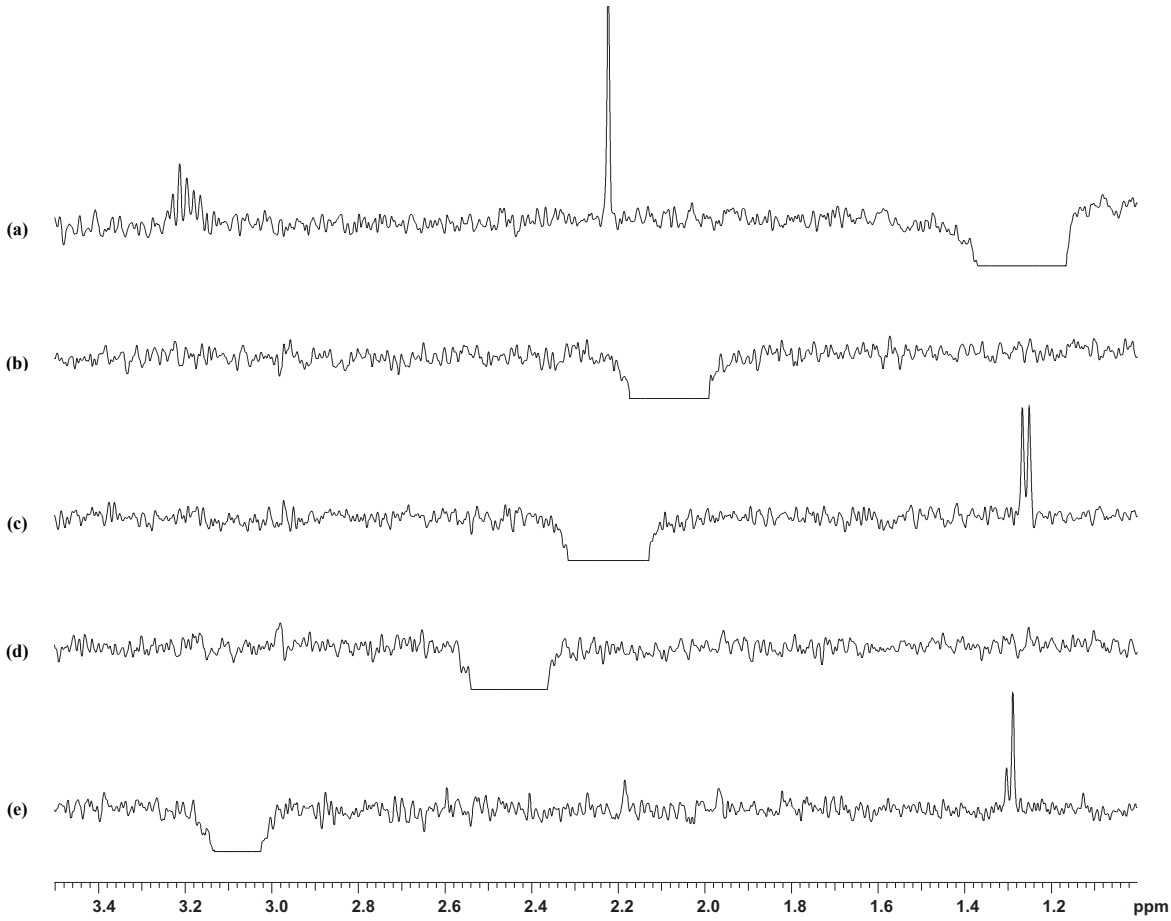


Figure 5.17: HMBC correlations of compound NAM 5-4.

The positions of the isopropyl and the carbonyl group were further confirmed by NOE experiment (Figures 5.18 (a) – (e)). When the methine proton C<u>H</u> (δ 3.1, m) and methyl proton C<u>H</u>$_3$ (δ 2.18, s) signals were irradiated, the signal of C<u>H</u>$_3$ (δ 1.29, d) was enhanced (Figures 5.18 (c) and (e), respectively). Irradiation of the methyl proton C<u>H</u>$_3$ (δ 1.29, d) enhanced the methine proton C<u>H</u> (δ 3.1, m) and methyl proton C<u>H</u>$_3$ (δ 2.18, s) signals (Figure 5.18 (a)). No signals were enhanced when the two methyl groups C<u>H</u>$_3$ (δ 2.07, s) and C<u>H</u>$_3$ (δ 2.41, s) were irradiated and these are shown in Figures 5.18 (b) and (d), respectively. These data from NOE experiment confirmed the relationship between the methine group (C-10, δ 31.6) and the two methyls (C-11, δ 20.7; C-12; δ 20.7) and the attachment overall of the isopropyl group at C-6 (δ 139.9).



Figures 5.18: 1D NOE NMR spectra for compound NAM 5-4 showing:

(a) Irradiation of C$\underline{H}_3$ (δ 1.29, d) signal, enhanced C$\underline{H}$ (δ 3.1, m) and C$\underline{H}_3$ (δ 2.18, s) signals;
(b) Irradiation of C$\underline{H}_3$ (δ 2.07, s), no signals were enhanced;
(c) Irradiation of C$\underline{H}_3$ (δ 2.18, s), enhanced C$\underline{H}_3$ (δ 1.29, d) signal;
(d) Irradiation of C$\underline{H}_3$ (δ 2.41, s), no signals were enhanced and;
(e) Irradiation of C$\underline{H}$ (δ 3.1, m) and C$\underline{H}_3$ (δ 2.18, s) signals, enhanced C$\underline{H}_3$ (δ 1.29, d) signal.

As previously stated, the mass of this compound was 255 Da ($[M+H]^+$). The ESIMS spectrum presented in Figure 5.13 also indicated the presence of an ion at m/z 207, which corresponded to the loss of 47 mass unit ($[M-SCH_3]^+$), thus a methyl thiol group was proposed. Confirmation of this group was supported by the HRESIMS$^+$ spectrum, which corresponded to a molecular formula of $C_{13}H_{19}O_3S$. The HRESIMS$^+$ of compound NAM 5-4 was recorded as 255.1043 Da and the calculated mass for $C_{13}H_{19}O_3S$ was 255.1055 Da (Δ = of -4.7 ppm).

At this point, the ^1H and ^{13}C chemical shifts for the proposed methyl thiol group of compound NAM 5-4 were compared with those for the two reported *S*-methyl benzothiates (**5.13** and **5.14**). Table 5.4 shows that chemical shifts of the methyl thiol group of compound NAM 5-4 were typical of compounds **5.13** (Tahara *et al.*, 1991) and **5.14** (Soman *et al.*, 1999). These data confirmed that the methyl thiol group (C-13, δ 11.4) was attached at the C-7 (δ 198.4).

Table 5.4: Comparison of ^1H NMR and ^{13}C NMR data for methyl thiol group of compounds NAM 5-4, **5.13** and **5.14**.

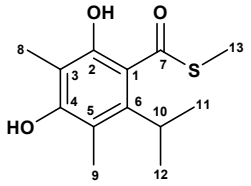
CH_3-S-C=O	**NAM 5-4[a]**		**5.13[b]**		**5.14[c]**	
	δ_H	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
	2.41, s	11.4	2.45, s	13.1	2.46, s	13.1
	-	198.4	-	198.2	-	198.3

[a] These data were recorded using CD_3OD; [b] These data were recorded using $CDCl_3$ (Tahara *et al*., 1991); [c] These data were recorded using $CDCl_3$ (Soman *et al*., 1999).

A complete list of NMR spectral data for NAM 5-4 is presented in Table 5.5. As there were no absolute matches for this structure found, the designated compound NAM 5-4 was therefore considered a new structure and named *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate (**5.15**).

Table 5.5: NMR data of compound NAM 5-4.

Position	δ ^{13}C, ppm		δ ^{1}H, ppm multiplicity (J_{HH} Hz)
1	122.2	(C)	
2	149.2	(C)	
3	110.2	(C)	
4	155.5	(C)	
5	115.5	(C)	
6	139.9	(C)	
7	198.4	(C)	
8	7.2	(CH_3)	2.07, s
9	12.1	(CH_3)	2.18, s
10	31.6	(CH)	3.1, m
11	20.7	(CH_3)	1.29, d (7.2)
12	20.7	(CH_3)	1.29, d (7.2)
13	11.4	(CH_3)	2.41, s



(5.15)

Part C: Phenolic carboxylic acid derivatives from F7089

5.5 Investigation of a large scale extract (F7089)

The second large scale extraction was carried out during the investigation of F5958-A as an attempt to obtain more of the metabolites in the cytotoxic region (15.0 – 18.5 min). The extraction of 118 plates gave a dark brown crude extract (99.8 mg) which re-named F7089. Bioactivity of F7089 was compared to that of the other two extracts (F5958 and F5958-A). The P388 assay showed that F7089 possessed slightly higher cytotoxicity (IC_{50}606 ng/mL), compared to F5958 (IC_{50}784 ng/mL), but lower than that for F5958-A (IC_{50}383 ng/mL).

The HPLC screening of extract F7089 showed that the attempt to obtain more of the desired metabolites within the cytotoxic region (15.0 – 18.5 min) from extract F7089 had been unsuccessful. The screening revealed three main compounds, NAM 5-2, NAM 5-5 and NAM 5-6 which were eluted over 8.0 – 12.5 min (Figure 5.19). The UV spectra of compounds NAM 5-2, NAM 5-5 and NAM 5-6 are shown in Figure 5.20. Based on these UV spectra, compound NAM 5-2 was in common with those of extracts F5958 and F5958-A as discussed in **Sections 5.3** and **5.4**. Compound NAM 5-2 was identified as bohemamine (**5.1**) (see **Section 5.3**). Compounds NAM 5-5 and NAM 5-6, however were additional compounds to those produced by F5958 (see Figure 5.2) and F5958-A (see Figure 5.8). Although compounds NAM 5-5 and NAM 5-6 were eluted over the inactive region, these compounds were further analyzed to establish the nature of the metabolite. Further investigation into compounds NAM 5-5 and NAM 5-6 is discussed below.

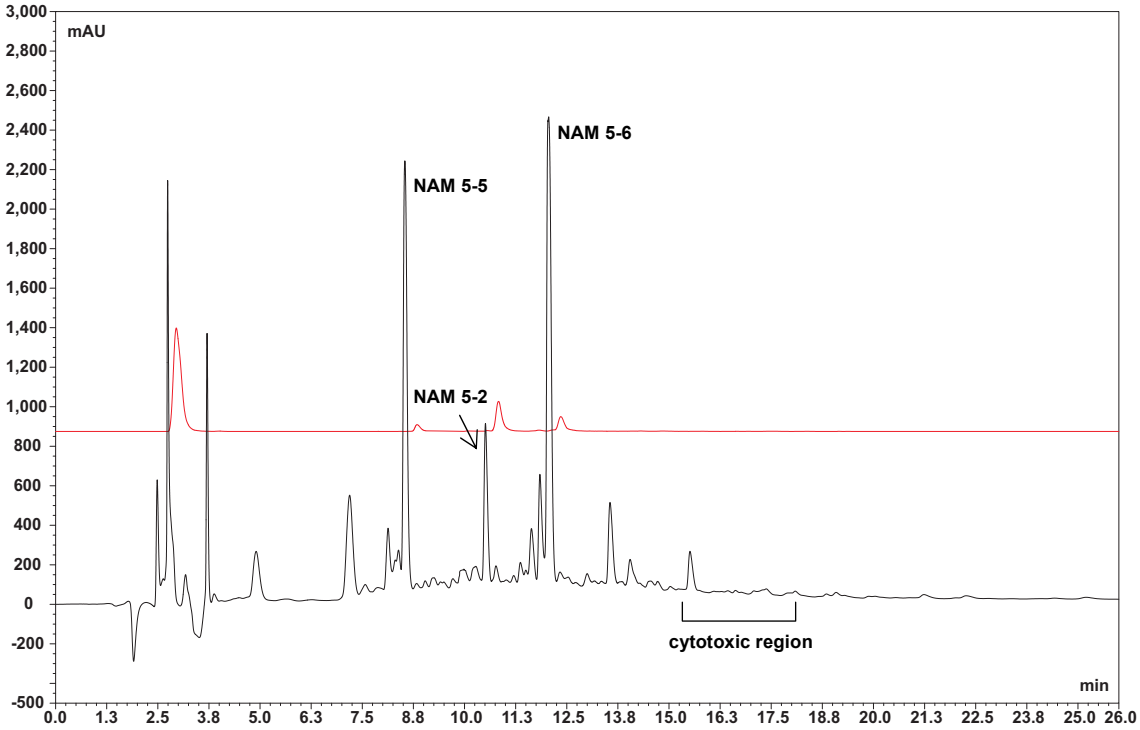


Figure 5.19: HPLC chromatogram of extract F7089 showing overlay of ELSD detection (top) for compounds NAM 5-2, NAM 5-5 and NAM 5-6.

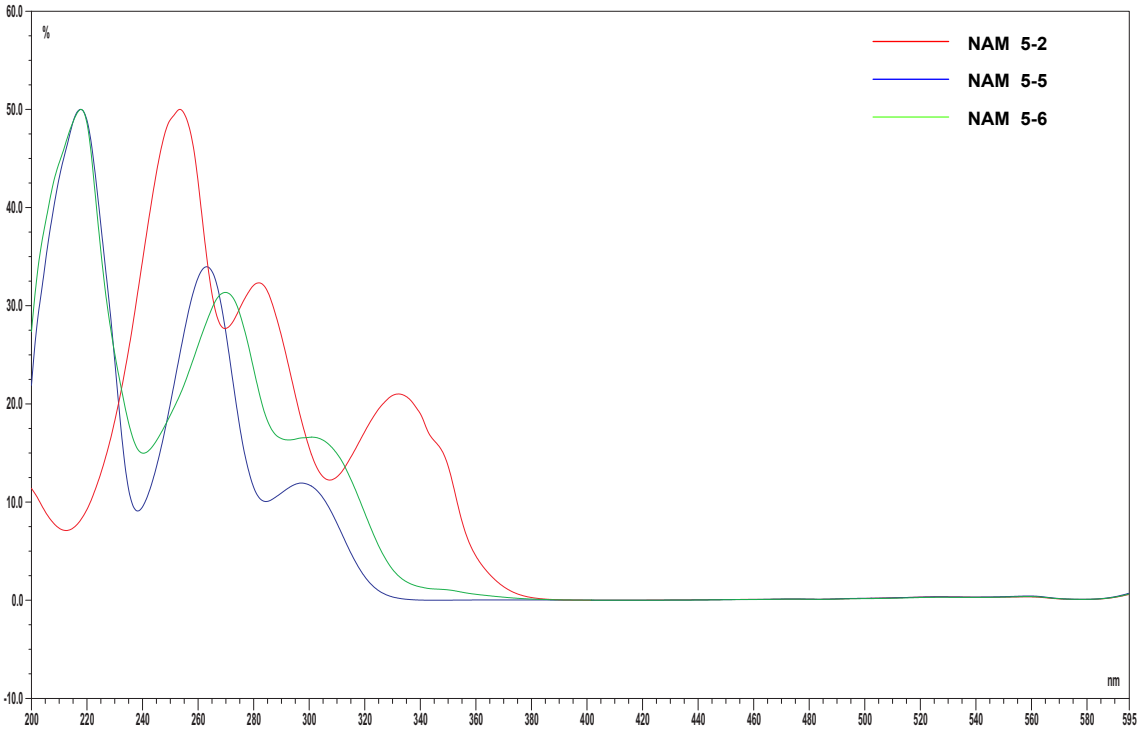


Figure 5.20: UV spectra overlay of compounds NAM 5-2, NAM 5-5 and NAM 5-6.

5.5.1 Isolation and purification of compounds NAM 5-5 and NAM 5-6

The crude extract F7089 (3 x 250 µg) was injected onto the HPLC and compounds NAM 5-5 and NAM 5-6 were collected into microtitre plates (see Figure 5.21). Both compounds were analyzed with the CapNMR technique and the structural elucidation is discussed below.

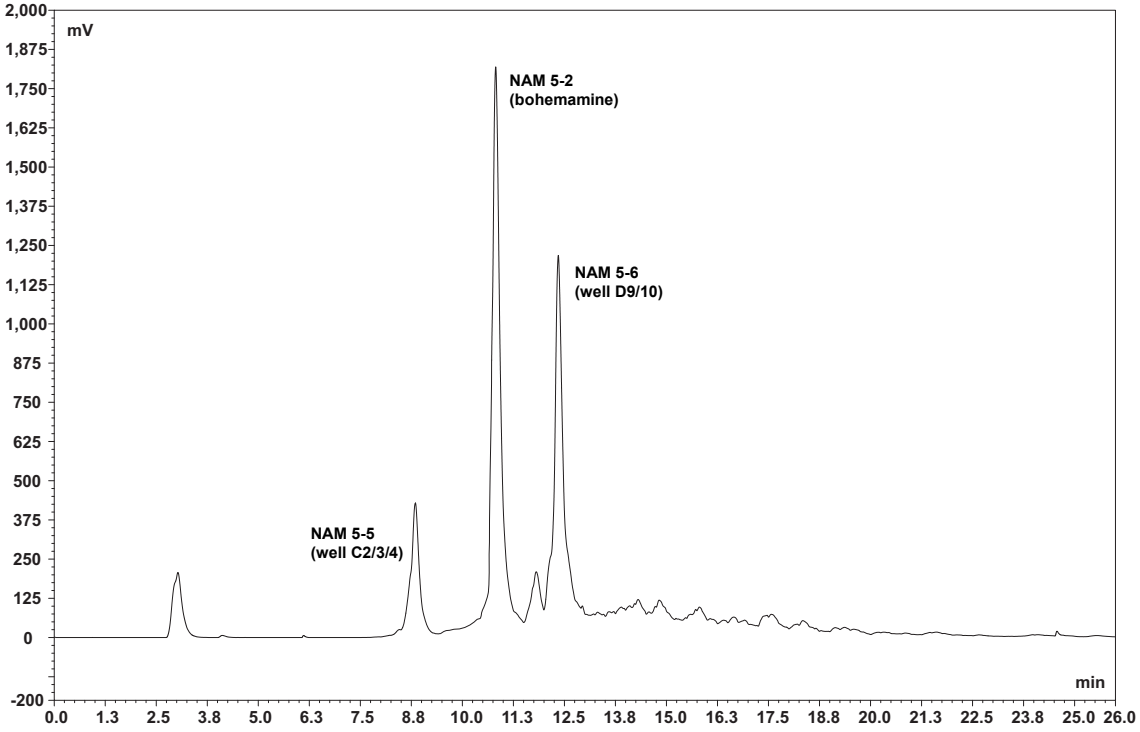


Figure 5.21: HPLC chromatogram of F7089 showing compounds NAM 5-5 and NAM 5-6 collected into wells of the HPLC microtitre plate.

5.5.2 Structural elucidation of NAM 5-5

An amount calculated to be approximately 8 μg of compound NAM 5-5 was analyzed by the CapNMR technique. The ^{1}H NMR spectrum (see Figure 5.22) revealed one singlet methyl (δ_H 2.53) and two aromatic protons at δ_H 6.15 and 6.22.

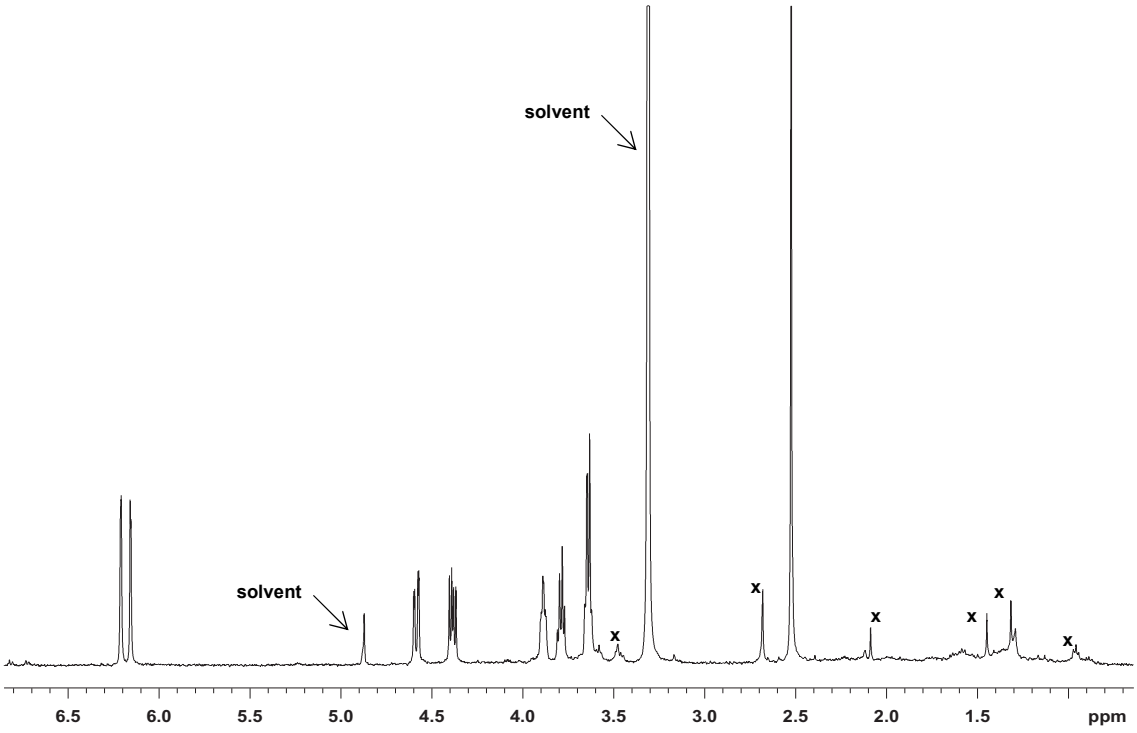


Figure 5.22: ^{1}H NMR spectrum of compound NAM 5-5 in CD_3OD obtained from F7089. Crosses indicate impurities.

The positive ion ESIMS (see Figure 5.23) suggested that the smaller signal at 273 Da was the parent ion ([M+H]), consistent with the main signal at 295 Da being the ([M+Na]) ion. The other peak that could be the parent ion ([M+H]) was 391 Da, which correlated well with a ([M+Na]) signal at 423 Da. None of the signals in the negative ion ESIMS could be rationalized as a parent ion, therefore the mass ([M+H]) of compound NAM 5-5 could possibly be 273 or 391 Da.

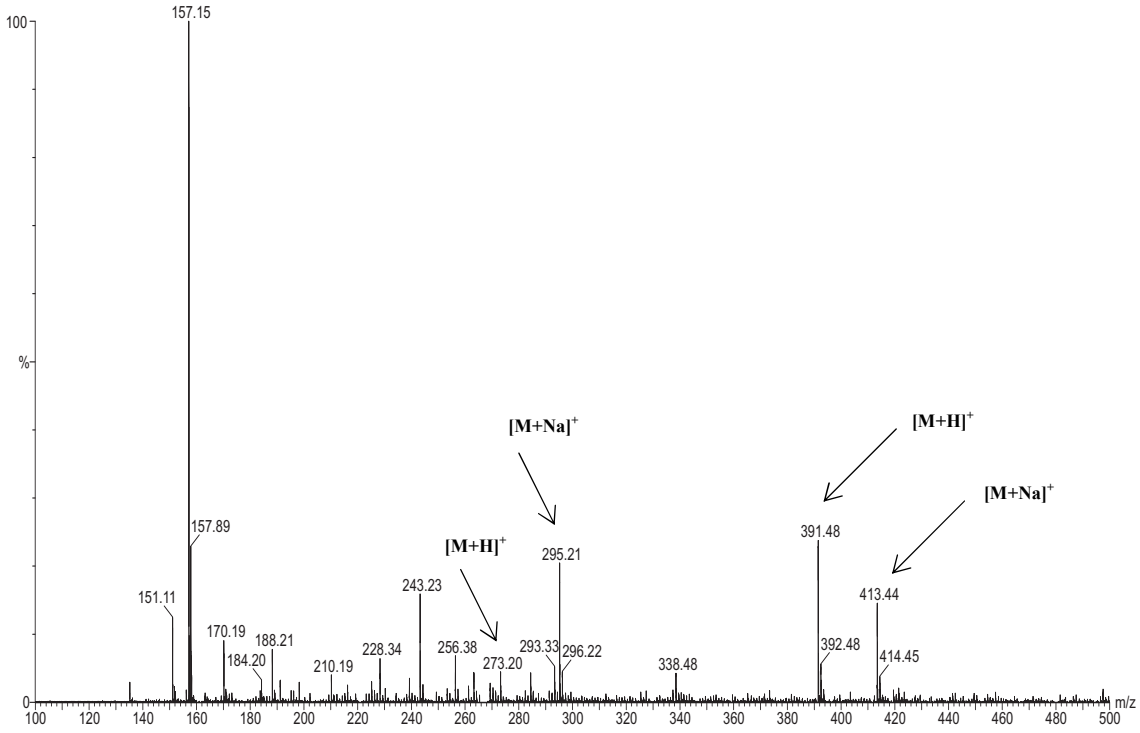


Figure 5.23: ESIMS spectrum of compound NAM 5-5.

A search in the AntiMarin database using the ^{1}H NMR and MS spectral data (one singlet methyl and molecular mass of 271 – 273 Da) resulted in ten hits. The *meta*-coupling pattern of the aromatic protons (δ_H 6.15 and 6.22) and the presence of the aromatic methyl in compound NAM 5-5 excluded nine of the ten known compound (see Figure 5.24). None of the nine hits obtained from the other search using the molecular mass of 389 – 391 Da matched with the ^{1}H NMR features of compound NAM 5-5.

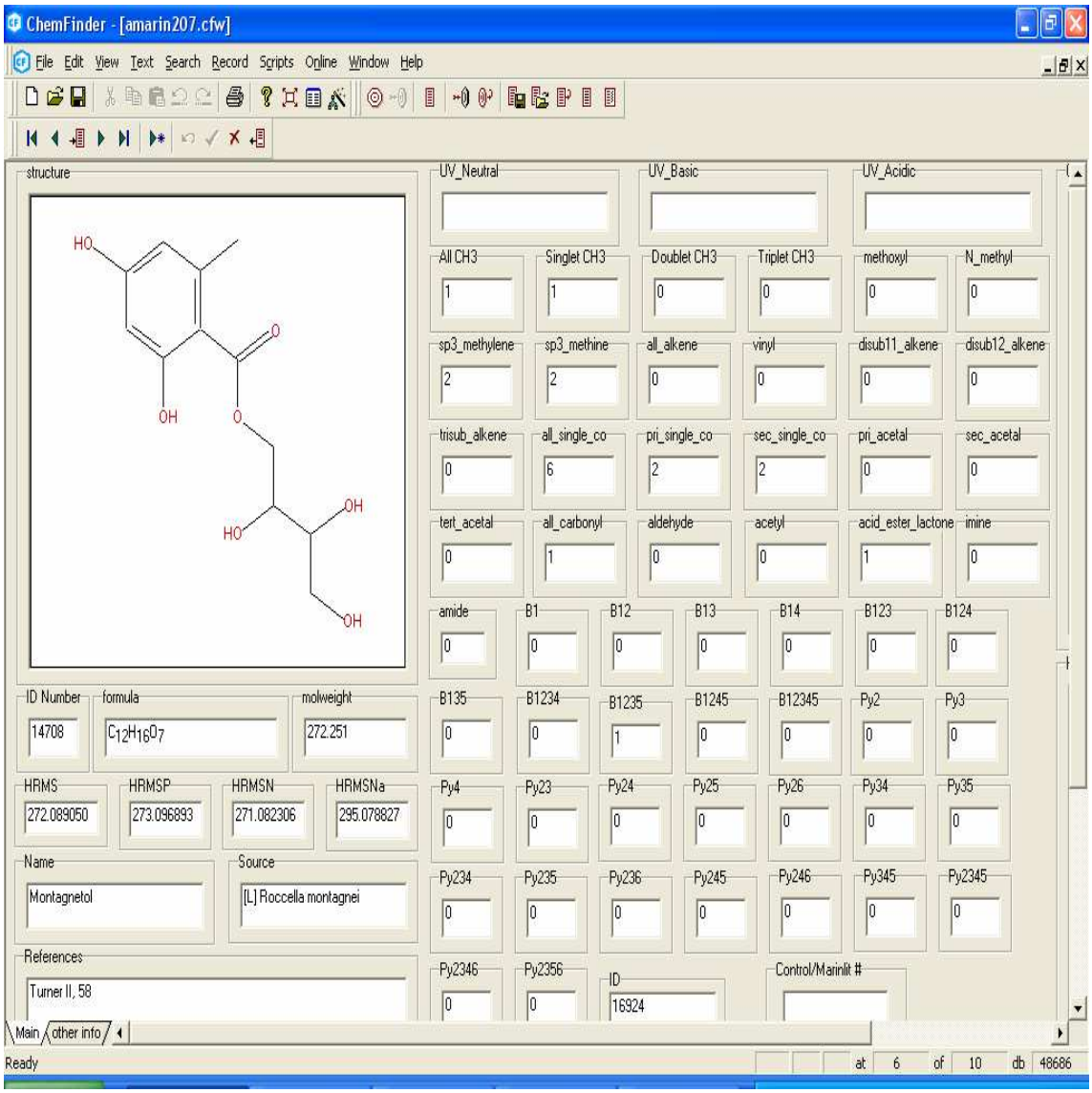
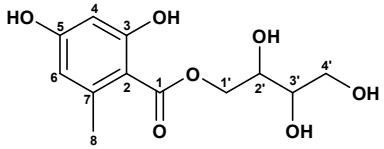
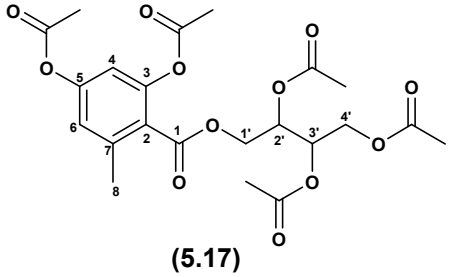


Figure 5.24: Screenshot of AntiMarin database search result for compound NAM 5-5 using the ^{1}H NMR and MS spectral data showing the hit with montagnetol (**5.16**).

The search showed that montagnetol (**5.16**) was the only possible known structure for compound NAM 5-5. This mono-aryl compound (**5.16**) was first isolated from the Indian lichen *Rocella montagnei* (Rao *et al*., 1941; Rao *et al*., 1942a; Rao *et al*., 1942b). This compound (**5.16**) was also reported from another lichen, *Ramalina hierrensis* (Gonzalez *et al*., 1992) where the structure of montagnetol (**5.16**) was deduced based on the ^{1}H NMR spectral data of montagnetol penta acetate (**5.17**) that was obtained after the acetylation of montagnetol (**5.16**).



(5.16)



(5.17)

The UV absorption of montagnetol (**5.16**) (Culberson, 1969) fits with that observed for compound NAM 5-5, however no publication on NMR spectral data for montagnetol (**5.16**) was available for comparison with the NMR data of compound NAM 5-5. Compound NAM 5-5 was then further analyzed with COSY, HSQC and HMBC experiments to give the complete characterization of this structure. These spectra are shown in Figures 5.25, 5.26 and 5.27, respectively.

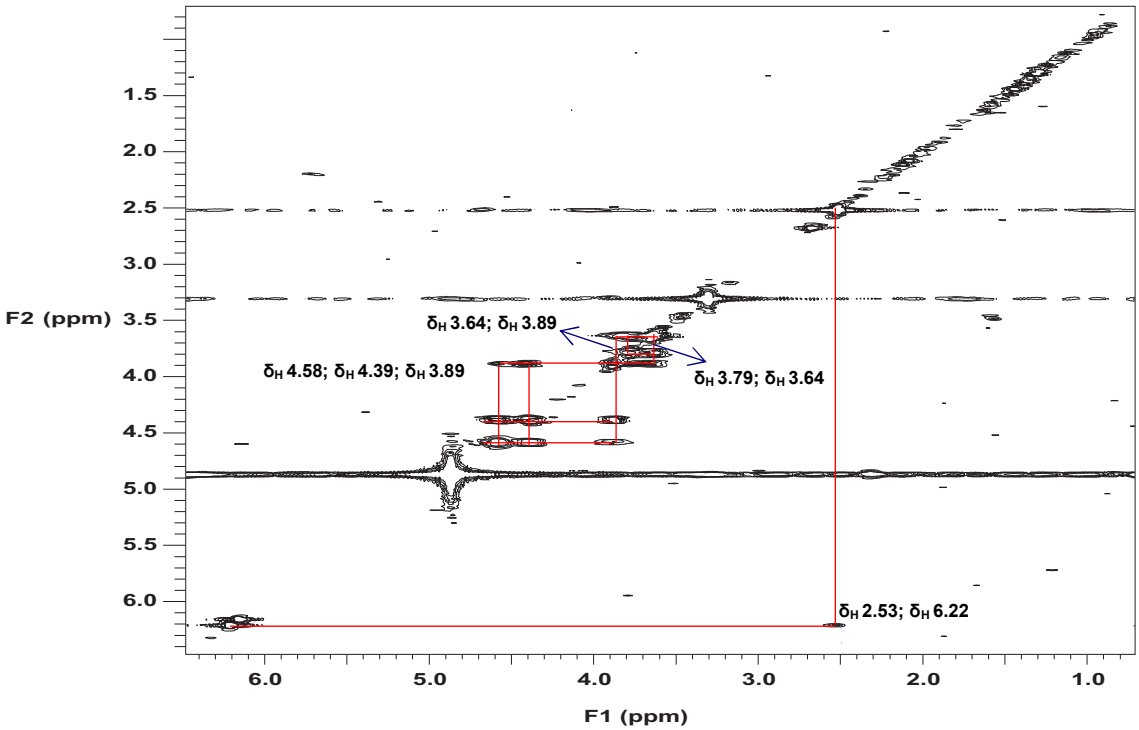


Figure 5.25: COSY spectrum of compound NAM 5-5.

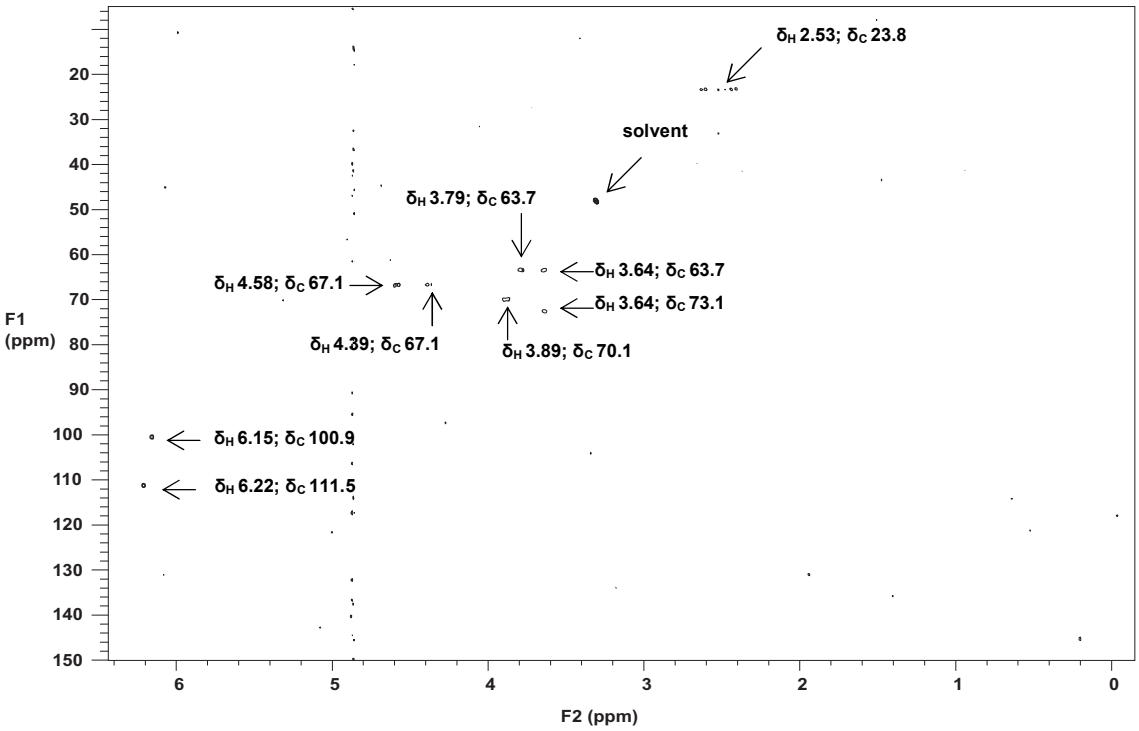


Figure 5.26: HSQC DEPT spectrum of compound NAM 5-5.

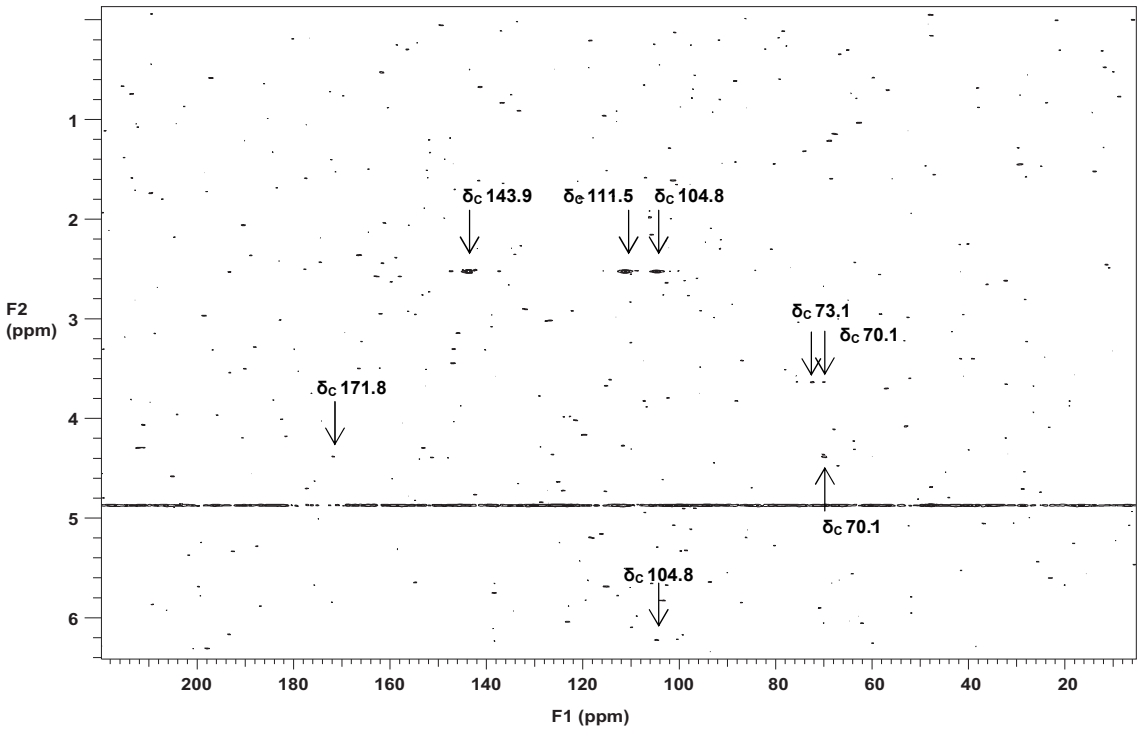
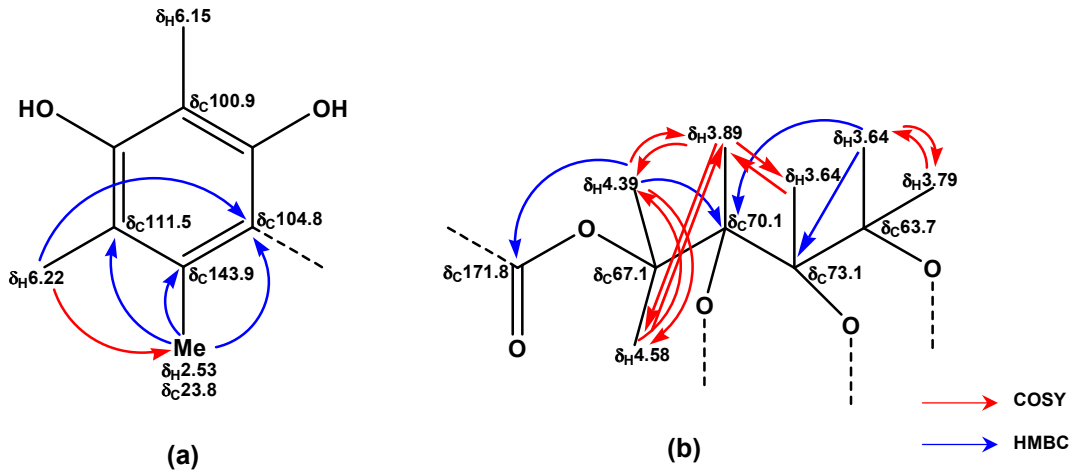


Figure 5.27: HMBC spectrum of compound NAM 5-5.

The *meta*-coupled aromatic protons at δ_H 6.15 and 6.22 were attached to carbons at δ_C 100.9 and 111.5, respectively (see Figure 5.26). The proton at δ_H 6.22 showed COSY correlation with the methyl protons at δ_H 2.53 and a long range correlation to the carbon at δ_C 104.8. The chemical shifts of three of the aromatic carbons (δ_C 100.9, 111.5 and 104.8) indicated the typical influence of phenolic groups. As the first two carbons (δ_C 100.9 and 111.5) were in *ortho* or *para*-relationship, the position of the third aromatic carbon (δ_C 104.8) could be deduced. Further, the position of the methyl group (δ_H 2.53; δ_C 23.8) was established by its strong $^3J_{CH}$ correlations with the aromatic carbons at δ_C 111.5 and 104.8 and a $^2J_{CH}$ correlation with the carbon at δ_C 143.9.

The presence of two -CH_2- groups in compound NAM 5-5 was indicated by the COSY and HSQC correlations. These were the protons at δ_H 3.64 and 3.79, both coupled to each other and attached to the same carbon at δ_C 63.7 and another two protons at δ_H 4.39 and 4.58 that were coupled to each other and both attached to the carbon at δ_C 67.1. The proton at δ_H 3.89 (δ_C 70.1) was coupled to both protons of the CH_2 group (δ_H 4.39 and 4.58) and to one proton at δ_H 3.64 (δ_C 73.1). This proton (δ_H 3.64; δ_C 73.1) in turn was coupled to both protons of the other CH_2 group (δ_H 3.64 and 3.79). Long range coupling was shown by one of the CH_2 proton (δ_H 3.64) to the carbons at δ_C 70.1 and 73.1. The HMBC spectrum also revealed the correlations of the other CH_2 proton (δ_H 4.39) to the carbons at δ_C 70.1 and to the carbon at δ_C 171.8. The chemical shifts of the carbons observed from the $^1J_{CH}$ correlations indicated the presence of four oxygenated carbons at δ_C 63.7, 67.1, 70.1 and 73.1. Since the CH_2 carbon (δ_C 67.1) was suggested to be oxygenated and had two protons (δ_H 4.39 and 4.58) attached to it, the long range correlation of the proton at δ_H 4.39 to the carbon at δ_C 171.8 had to be a $^3J_{CH}$ correlation, thus confirming the position of the carbon at δ_C 171.8. Based on these data, two fragments of the structure of compound NAM 5-5 were suggested as illustrated in Figures 5.28 (a) and (b).



Figures 5.28 (a) and (b): Two fragments of compound NAM 5-5 deduced from COSY, HSQC and HMBC correlations.

In the first fragment presented in Figure 5.28 (a), the chemical shift of the methyl group at δ_H 2.53 suggested that the methyl group was in a *peri*-position with respect to the carbonyl group. Therefore, the carbonyl group (δ_C 171.8) had to be adjacent to the aromatic carbon at δ_C 104.8. Based on the assumption that all three oxygenated carbons (δ_C 63.7, 70.1 and 73.1) in the second fragment (Figure 5.28 (b)) were attached to hydroxyl, the structure of compound NAM 5-5 fits with those reported for montagnetol (**5.16**).

The ACD/Labs prediction software was then used to simulate the ^{1}H and ^{13}C NMR chemical shifts of montagnetol (**5.16**) for comparison with the experimental data obtained for compound NAM 5-5 (see Table 5.6). The comparison showed that the calculated data for montagnetol (**5.16**) and compound NAM 5-5 were almost identical, suggesting that compound NAM 5-5 was indeed montagnetol (**5.16**).

Table 5.6: Comparison of ^{1}H and ^{13}C NMR data of compound NAM 5-5 and of the simulated values of montagnetol (**5.16**).

Position	**NAM 5-5** [b]		**5.16** [a]	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1 (-C=O)		171.8		171.2
2		104.8		104.9
3 (-OH)				165.9
4	6.15	100.9	6.15	102.5
5 (-OH)				161.5
6	6.22	111.5	6.19	112.0
7		143.9		144.9
8	2.53	23.8	2.48	24.7
1'	4.39; 4.58	67.1	4.35; 4.49	65.4
2'	3.89	70.1	4.45	70.4
3'	3.64	73.1	3.62	72.9
4'	3.64; 3.79	63.7	3.62; 3.76	62.8

[a] These data were simulated by ACD/Labs software, version 10; [b] These data were recorded at 500 MHz in CD_3OD.

5.5.3 Structural elucidation of NAM 5-6

The ^{1}H NMR spectrum of compound NAM 5-6 (Figure 5.29) indicated identical proton signals at δ_H 2.5 – 6.5 to those of compound NAM 5-5 (see Figure 5.22). Based on the integrals, compound NAM 5-6 contained an additional methyl group at δ_H 2.57 and additional two aromatic protons at δ_H 6.67, compared to those of compound NAM 5-5 (see Figure 5.22). The structural features of compound NAM 5-5 (**5.16**) were then used as a basis to identify compound NAM 5-6.

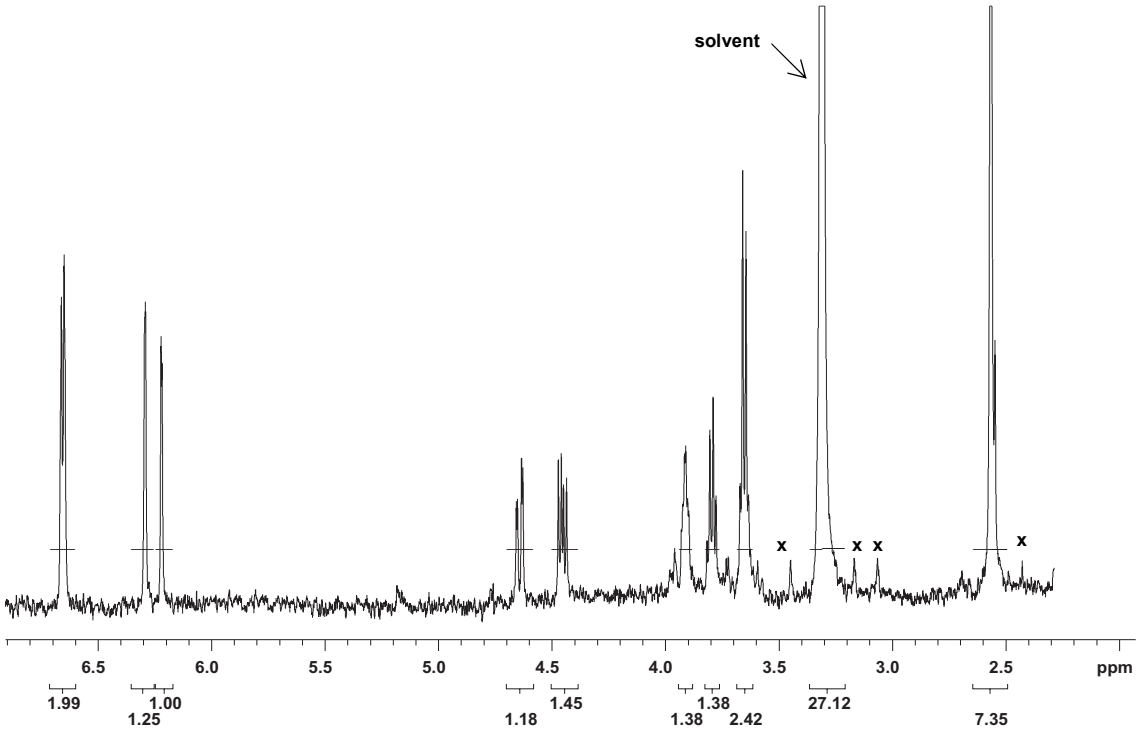


Figure 5.29: ^{1}H NMR spectrum of compound NAM 5-6 in CD_3OD obtained from F7089. Crosses indicate impurities.

Compound NAM 5-6 was shown to have a molecular weight of 421.81 Da based on the presence of ion signal at 420.81 Da $([M-H])^-$ (see Figure 5.30).

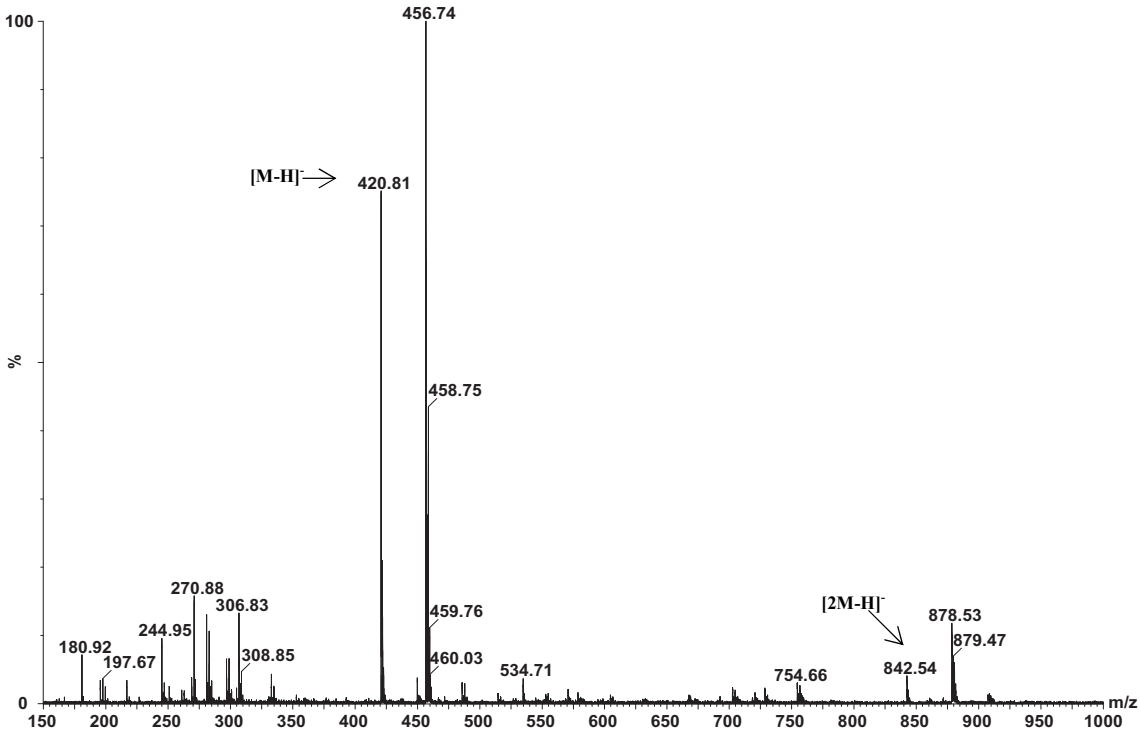
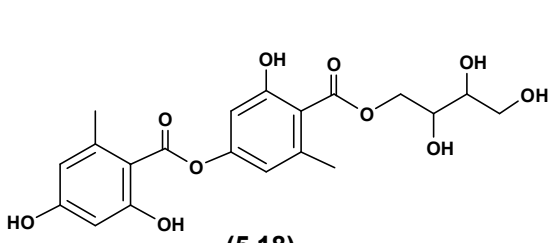
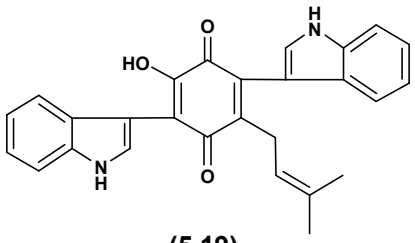


Figure 5.30: ESIMS spectrum (negative ionization) of compound NAM 5-6.

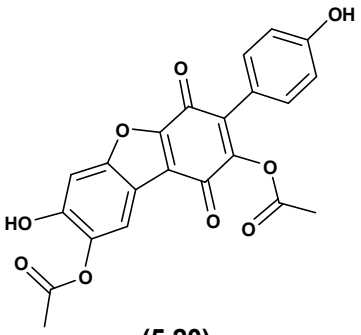
A search in the AntiMarin database using the molecular weight of 421 – 423 Da and two methyl groups led to four hits. These were erythrin (**5.18**), ochrindole-D (**5.19**), anthracophyllin (**5.20**) and thaxtomin B (**5.21**). Based on the structural features of compound NAM 5-5 assigned as montagnetol (**5.16**), erythrin (**5.18**) was found to be the only possible structure for compound NAM 5-6. The UV absorption of erythrin (**5.18**) was reported having UV (MeOH), λ_{max} (log ε) mμ: 269 (4.0) and 303 (4.3) (Culberson, 1969). This data was almost identical to those observed for compound NAM 5-6 as presented in Figure 5.20.



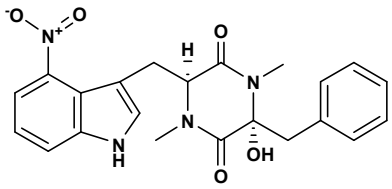
(5.18)



(5.19)



(5.20)



(5.21)

Erythrin (**5.18**), like montagnetol (**5.16**) was also isolated from the Indian lichen *Rocella montagnei* in the 1940s (Rao *et al*., 1942b). Erythrin (**5.18**) was then reported from other lichens, *Opegrapha platycarpa* (Huneck *et al*., 1968a) and *Dirina* spp. (Huneck *et al*., 1968b). As there was no published NMR spectral data for erythrin (**5.18**), COSY and HSQC experiments were performed to allow structural elucidation of compound NAM 5-6. The COSY and HSQC spectra are shown in Figures 5.31 and 5.32, respectively.

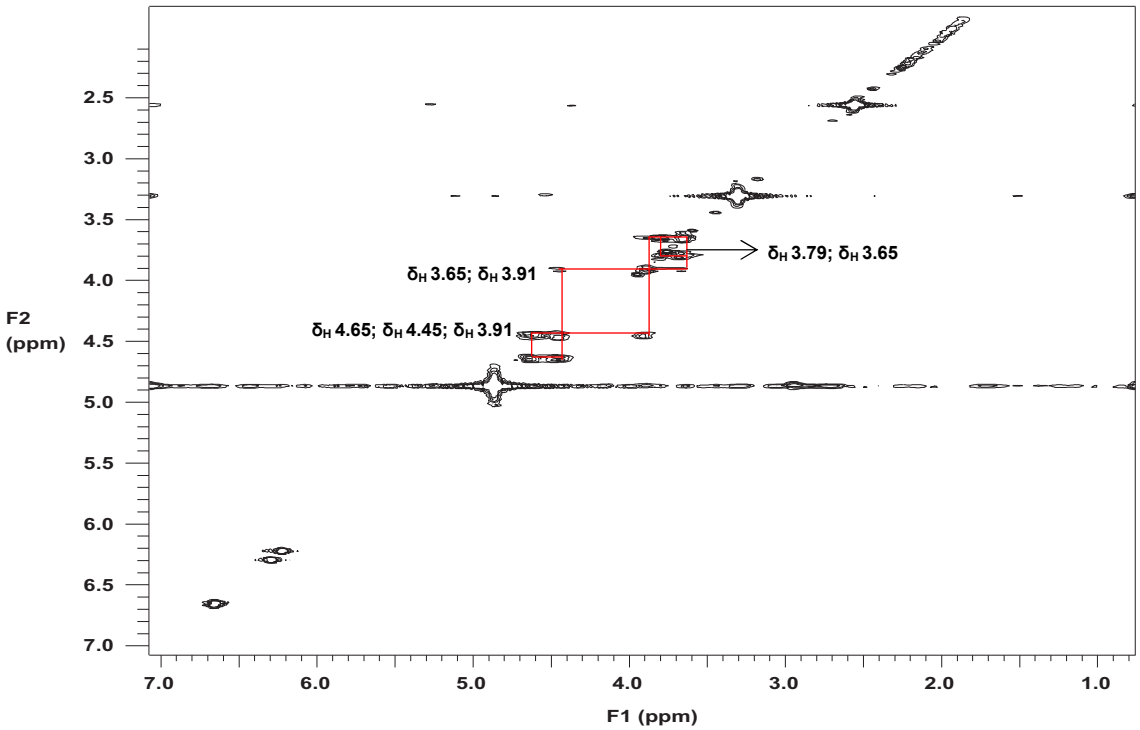


Figure 5.31: COSY spectrum of compound NAM 5-6.

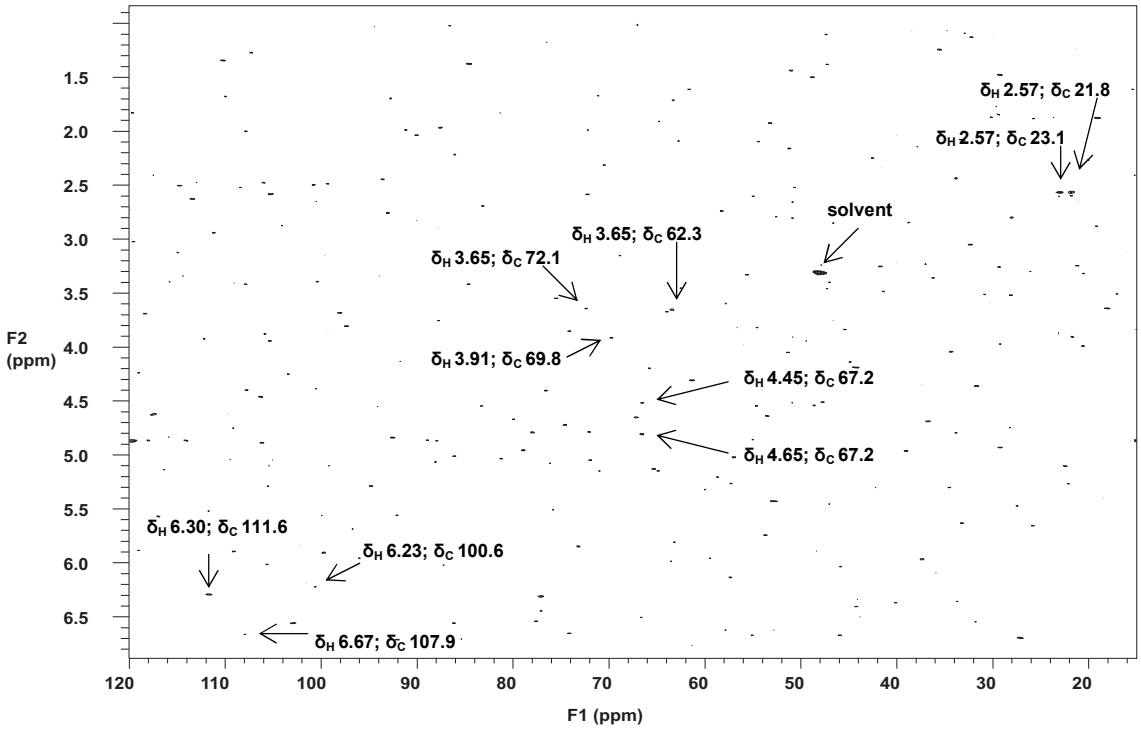


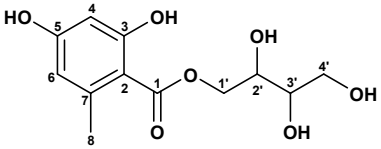
Figure 5.32: HSQC DEPT spectrum of compound NAM 5-6.

The data for compound NAM 5-6 were compared with that of compound NAM 5-5 (**5.16**) and the ACD/Labs software simulation for erythrin (**5.18**) (see Table 5.7). The ^{1}H and ^{13}C NMR data of compound NAM 5-6 at position C-1', C-2', C-3' and C-4' were consistent with compound NAM 5-5 (**5.16**) and the calculated value for erythrin (**5.18**). The comparison between the experimental data for compound NAM 5-6 showed that the calculated data for erythrin (**5.18**) were almost identical, leading to the assignment of compound NAM 5-6 as erythrin (**5.18**).

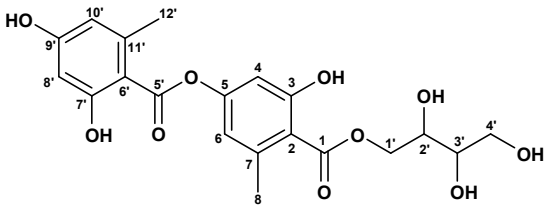
Table 5.7: Comparison of ^{1}H and ^{13}C NMR data of compound NAM 5-6, compound NAM 5-5 and the simulated values of erythrin (**5.18**).

Position	NAM 5-6 [a]		NAM 5-5 [a]		5.18[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
4	6.67	107.9	6.15	100.9	6.79	107.75
6	6.67	107.9	6.22	111.5	6.56	113.70
8	2.57	23.1	2.53	23.8	2.77	24.27
1'	4.45; 4.65	67.2	4.39; 4.58	67.1	4.35; 4.49	68.40
2'	3.91	69.8	3.89	70.1	4.45	70.40
3'	3.65	72.1	3.64	73.1	3.62	73.11
4'	3.65; 3.79	62.3	3.64; 3.79	63.7	3.62; 3.76	64.40
8'	6.23	100.6	na	na	6.27	112.80
10'	6.30	111.6	na	na	6.21	101.7
12'	2.57	21.8	na	na	2.48	24.27

[a] These data were recorded at 500 MHz in CD_3OD; [b] These data were simulated by ACD/Labs software, version 10; na: not applicable.



(5.16)



(5.18)

Part D: Effect of temperature and salinity on growth and cytotoxicity of *Streptomyces* sp. (LA3L2)

5.6 Initial screening

The cultivation conditions of *Streptomyces* sp. (LA3L2) used for preliminary screening (SCA medium, pH 7.0±0.2; 4% salinity) were used as a basis to determine its optimum temperature for growth. Growth as colony diameter was measured at 0, 7, 10, 17, 20, 24, 28, 32, 36, 40, 44 and 50°C every two days for 30 days. Details of the procedures are given in **Chapter Two** (*Experimental*).

The growth of *Streptomyces* sp. (LA3L2) after 10, 20 and 30 days incubation is shown in Figure 5.33. Growth occurred between 10 and 40°C with an optimum of 32 – 36°C. No growth was observed at 0, 7 or at 45°C and above. An experiment to investigate the interaction of temperature and salinity on growth was conducted at five temperatures (20, 24, 28, 32 and 36°C) where reasonable growth was attained.

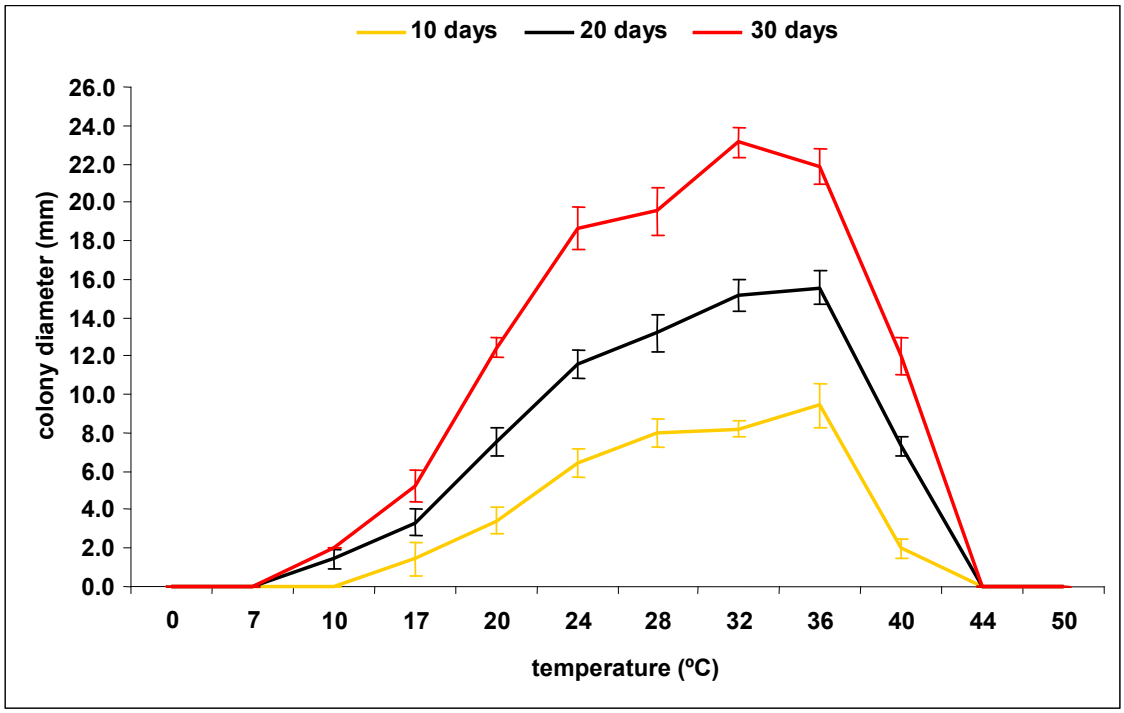


Figure 5.33: Effect of temperature on growth on *Streptomyces* sp. (LA3L2) on SCA medium (pH 7.0±0.2; 4% salinity) after 10, 20 and 30 days incubation.

5.7 Effect of temperature and salinity on growth

Salinity (0, 2, 4, 6, 8 and 10%) was tested at five temperatures (20, 24, 28, 32 and 36ºC) on SCA medium (pH 7.0±0.2). Growth diameter was measured for every 2 days for 30 days. Details of the procedures are discussed in **Chapter Two** (*Experimental*).

The results after 30 days incubation are shown in Figures 5.34. The isolate grow at all sea salt concentrations tested. At all temperatures growth increased up to 6% salinity and at the lowest incubation temperature (20°C) growth increased to 8% salinity.

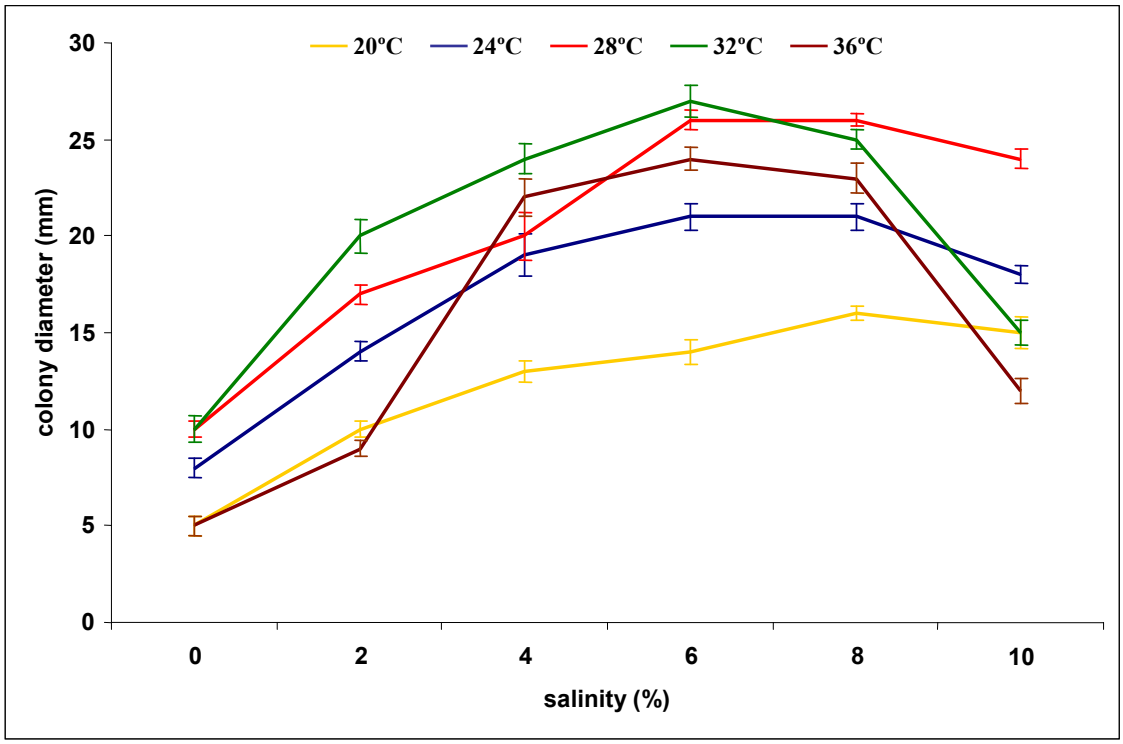


Figure 5.34: Effect of salinity on growth on *Streptomyces* sp. (LA3L2) on SCA medium (pH 7.0±0.2) at 20, 24, 28, 32 and 36ºC after 30 days incubation.

5.8 Effect of temperature and salinity on cytotoxicity

Cultures from the effect of temperature and salinity on growth experiment were harvested after 30 days incubation and extracted. The extracts (1 mg/mL) were assayed for cytotoxicity against P388 cells and their metabolite profiles assessed by HPLC. The metabolites were confirmed by their UV characteristics as described in **Section 5.3** (bohemamine and bohemamine B), **Section 5.4** (*S*-methyl benzothioate), **Section 5.5** (montagnetol and erythrin). Details of the procedures are discussed in **Chapter Two** (*Experimental*).

The results of the effect of temperature and salinity on cytotoxicity are shown in Table 5.8. All extracts were active against the P388 cells at all temperatures and salinity tested. Cytotoxicity was best at a temperature of 24ºC and 4% salinity (see Figure 5.35). Cytotoxicity increased with increasing salinity up to 4% but in most instances the activity decreased at higher salinity levels.

Table 5.8: Effect of salinity on cytotoxicity of *Streptomyces* sp. (LA3L2) on SCA medium (pH 7.0±0.2; 30 days).

Salinity (%)	**Cytotoxicity against P388 cells (IC_{50} ng/mL) Temperature (ºC)**				
	20	24	28	32	36
0	712	296	372	1339	1225
2	513	138	187	783	783
4	296	<97.5	123	655	783
6	857	516	501	712	783
8	1225	599	501	1055	783
10	6381	857	1055	2906	4154

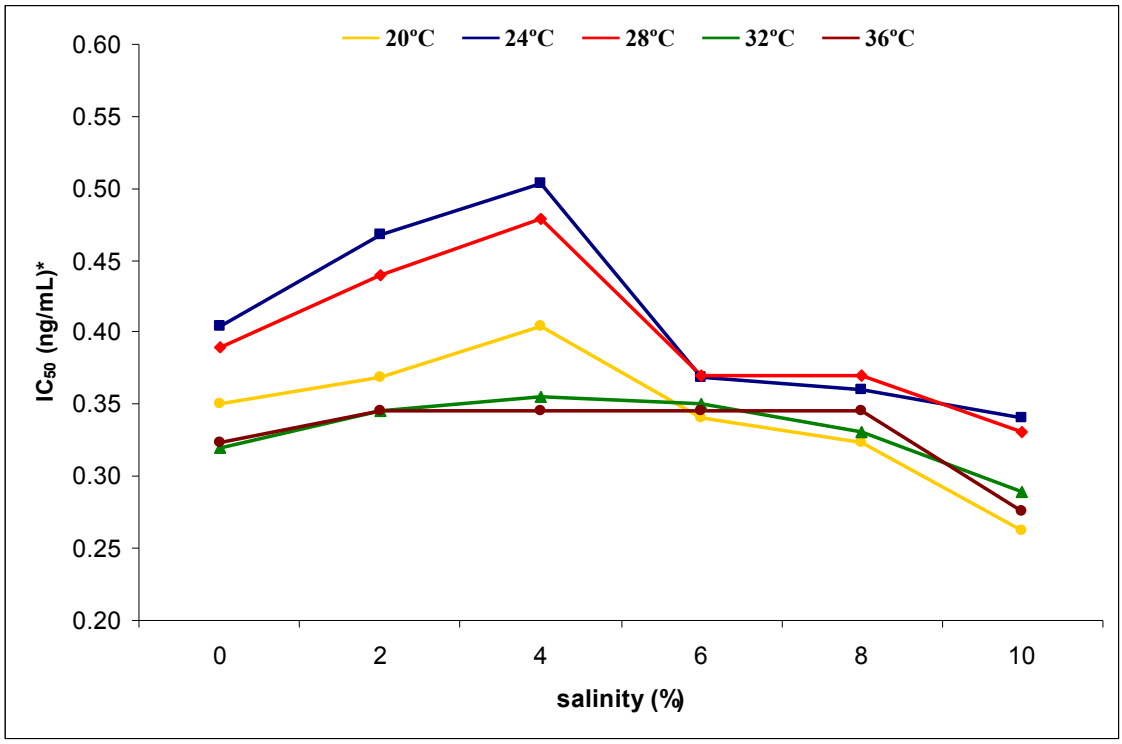


Figure 5.35: Effect of temperature and salinity on cytotoxicity of *Streptomyces* sp. (LA3L2) cultured on SCA medium (pH 7.0±0.2) after 30 days incubation. *IC_{50} (ng/mL) was expressed in log values (1/log IC_{50} value).

The results of the HPLC profiles (see Figure 5.36) are represented by five extracts obtained from isolates cultured at 4% salinity at five temperatures (20, 24, 28, 32 and 36ºC). Although all five extracts showed cytotoxicity below 1,000 ng/mL, none of the ELSD traces in the HPLC chromatogram showed significant production of metabolites in the cytotoxic region (15.0 – 18.5 min). The presence of cytotoxic *S*-methyl benzothioate (NAM 5-4) was significantly detected from the UV traces of one extract (24ºC) (see Figure 5.36). The remaining extracts (20, 28, 32 and 36ºC) showed only minor UV traces of the metabolite. Bohemamine was the only metabolite that was significantly produced at 20, 24 and 28ºC, but not at higher temperatures (32 and 36ºC). Bohemamine B, montagnetol and erythrin was not produced in significant amount in any of the five extracts.

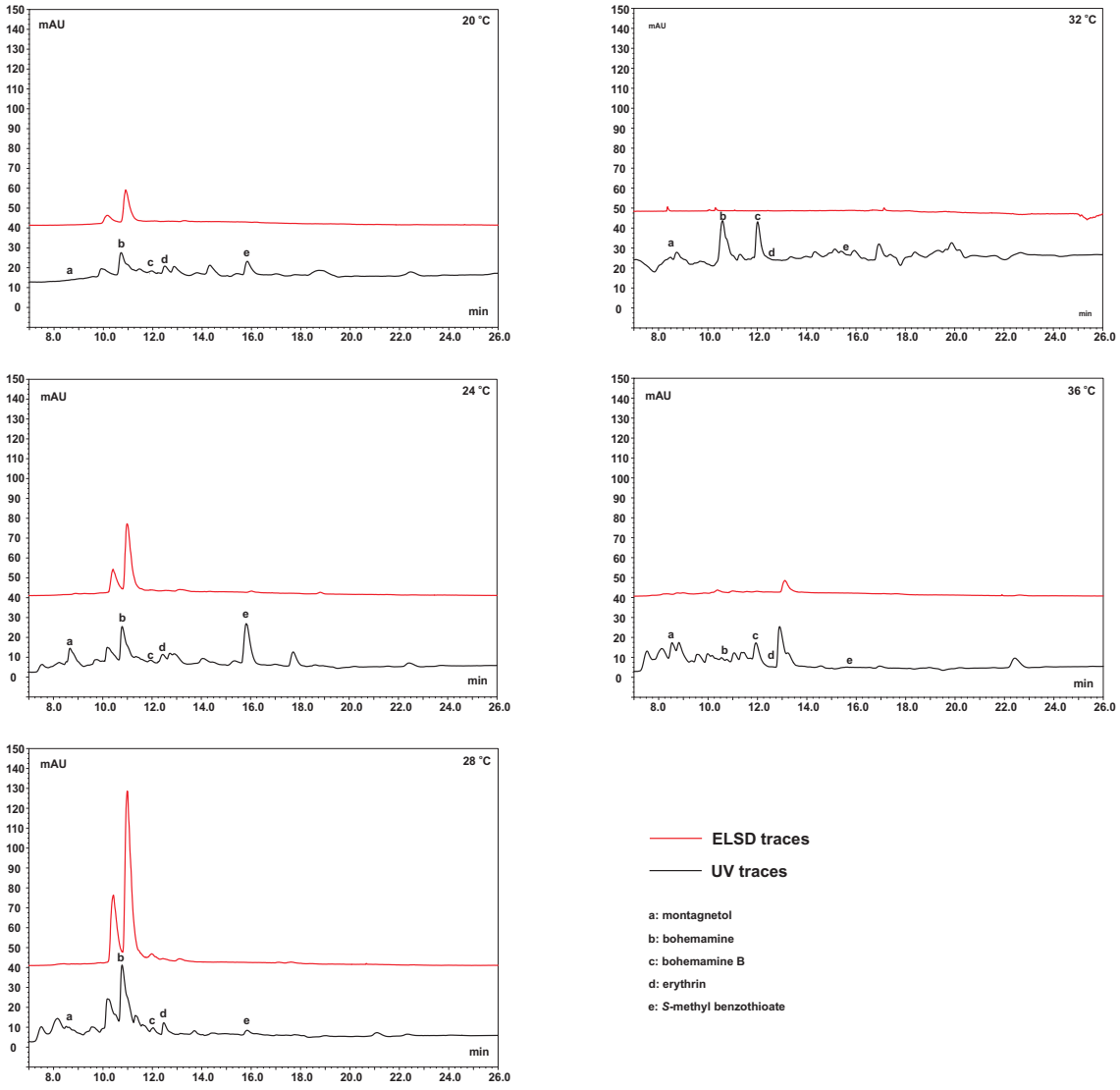


Figure 5.36: HPLC chromatogram of five extracts obtained from *Streptomyces* sp. (LA3L2) cultured on SCA medium (pH 7.0±0.2; 4% salinity) at 20, 24, 28, 32 and 36ºC showing traces of metabolites.

5.9 Discussion

Large scale cultivations of *Streptomyces* sp. (LA3L2) were undertaken to generate production of bioactive metabolites for structure elucidation. *Streptomyces* sp. (LA3L2) was targeted for large scale cultivation because it showed good activity against P388 cells (IC_{50}784 ng/mL), however, active metabolites were not significantly produced. It became apparent in the second large scale cultivation that isolate LA3L2 was inconsistent in producing the desired bioactive metabolites, but did show consistent production of other compounds.

This isolate produced bohemamine significantly in both the small and large scale cultivations, while bohemamine B was inconsistently produced in these conditions. The bohemamines are a rare pyrrolizidines group of compounds with an unusual methylation pattern and presence of amide nitrogen. To date, bohemamines have only been reported from actinomycetes. Bohemamine was isolated from *Actinosporangium* sp. (Doyle *et al*., 1980; Nettleton *et al*., 1980); bohemamine, bohemamine B, bohemamine C and 5-chlorobohemamine C from a marine-derived *Streptomyces* sp. (Bugni *et al*., 2006); NP25302 from *Streptomyces* sp. (Zhang *et al*., 2003b) and three metabolites with a similar pyrrolizidine ring system from a *Streptomyces* sp. (Hu *et al*., 2003). Bohemamine and bohemamine B isolated from LA3L2 were found to be inactive in both the cytotoxic and antimicrobial assays. Bugni *et al.* (2006) similarly found bohemamine and bohemamine B inactive in the HCT-116 colon carcinoma cell line and antimicrobial assays.

The first large scale cultivation revealed small amounts of a new cytotoxic metabolite - *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate. This metabolite was determined as one of the cytotoxic components from HPLC MTT plate assay but was inactive against bacteria or fungi. Tahara *et al.* (1991) reported the production of resorthiomycin (*S*-methyl 2,4-dihydroxy-5-(3-hydroxybutyl)-3,6-dimethylbenzothioate) from a soil-derived *S. collinus* which exhibited cytotoxicity against mouse leukemia cells and the human hepatoma cell line but was inactive against bacteria and fungi (Suzuki *et al*., 1990). Mortivinacin A (*S*-methyl 2,4-dihydroxy-3,5,6-trimethylbenzothioate) was reported from a sclerotium-colonizing isolate of the fungus, *Mortierella vinacea* and was active against *C. albicans*, *B. subtilis* and *S. aureus* (Soman *et al*., 1999). The new metabolite is thus only the third compound of this group to be characterized.

The attempt to obtain other cytotoxic metabolites in the second large scale cultivation was unsuccessful. Two other different metabolites, however, were produced and characterized by CapNMR. These were montagnetol and erythrin, two lichen-related metabolites. Both montagnetol and erythrin were first isolated from the Indian lichen *Rocella montagnei* (Rao *et al*., 1942b). Montagnetol was then reported from another lichen, *Ramalina hierrensis* (Gonzalez *et al*., 1992) while erythrin was isolated from other lichens; *Opegrapha platycarpa* (Huneck *et al*., 1968a) and *Dirina* spp. (Huneck *et al*., 1968b). Structural similarities between natural products of fungal origin are common with those of lichens such as averythrin from the lichen *Solorina crocea* (Yutaka *et al*., 1970) and the fungus *Herpotrichia rhodosticta* (Van Eijk and Roeijmans, 1984). The depsidones were reported from lichens such as *Erioderma phaeorhizum* (Bin Hamat *et al*., 1992); *Phaeographis* sp. (Elix *et al*., 2003) and *Usnea* sp. (Kathirgamanathar *et al*., 2005) and also from fungi such as an unidentified endophytic fungus (Pittayakhajonwut *et al*., 2006) and the mangrove-derived fungus *Preussia aurantiaca* (Poch and Gloer, 1991). Similarities between secondary metabolites originating from actinomycetes with those of lichens have not been previously reported.

Inconsistent metabolite production in suceeding fermentations is often seen and presents problems for commercialization of metabolites. Both genetic and environmental causes have been cited for such occurrences. *Streptomyces* sp. (LA3L2) was a marine-derived organism and in an attempt to determine the possible effects of environmental parameters such as salinity and temperature on growth and metabolite production a series of experiments was conducted. Isolate LA3L2 was shown to be able to grow on media containing up to 10% sea salt. Growth of *Streptomyces* spp. appears to be variable in respect to salinity. A *Streptromyces* sp. derived from the sponge, *Dendrilla nigra* (Selvin *et al*., 2004) showed growth up to 8% NaCl concentration, but did not grow at 10% NaCl concentration. It has been reported that a strain of *Streptomyces* sp., isolated from shallow sea mud failed to grow at 15ºC in medium containing 3.5% NaCl (Okami, 1974). The influence of NaCl concentration and temperature on growth and metabolite production in *Streptomyces* spp. has been demonstrated in only a few studies. Sultan *et al.* (2002) found that 2% NaCl concentration and 37.5ºC were the optimum cultivation conditions for antibacterial metabolite production in a soil-derived *Streptomyces* sp. The effect of salinity on metabolite production in *S. avermitilis* showed variations. The content of palmitoleic acid in *S. avermitilis* increased with increasing salinity up to 12% NaCl concentration but avermectin production decreased after 0.5% NaCl concentration (Rezanka and Votruba, 1998).

Streptomyces sp. (LA3L2) showed that after 30 days incubation, the optimum temperature for growth (32 - 36ºC) was higher than the optimum temperature for cytotoxic metabolite production (24ºC). This is consistent with the typical effect of temperature on growth and metabolite production by mesophilic actinomycetes (Votruba and Vanek, 1989). The temperature optimum for secondary metabolism is often lower than that for primary metabolism and replicative growth of the producing organism (Weinberg, 1974). The cultivation of microorganisms at sub-optimal temperatures decreases the demand for substrate supporting primary metabolism (Farrel and Rose, 1967; Demain, 1968; Votruba and Vanek, 1989). The influence of temperature on maximal and minimal yield of secondary metabolites in some species of *Streptomyces* is shown in Figure 5.37.

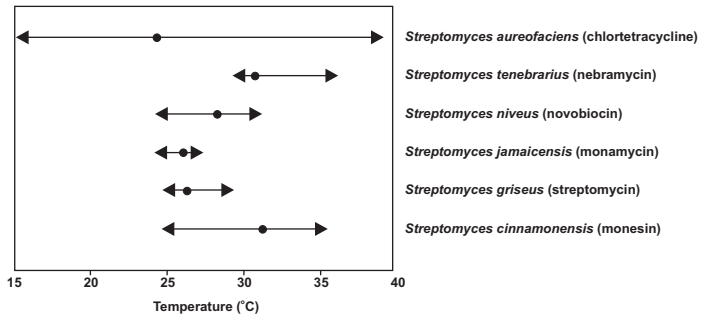


Figure 5.37: The influence of temperatures permitting vegetative growth on secondary metabolism. Circle: temperature at which maximal yield of secondary metabolite is obtained; arrow tips: temperature at which minimal yields of secondary metabolite are obtained (Adapted from Votruba and Vanek (1989)). Physicochemical factors affecting actinomycete growth and secondary metabolism. In Shapiro, S. (ed.). Regulation of secondary metabolism in actinomycetes. CRC Press. Inc. p 274).

Cultural conditions of 24ºC and 4% salinity led to an extract showing greatest cytotoxicity (IC_{50}<97.5 ng/mL), however, ELSD traces of this extract did not show significant traces of the characterized cytotoxic metabolites. Secondary metabolite production in microbes is strongly influenced by growth medium (such as carbon and nitrogen sources and micronutrients) and cultural conditions. These factors need to be examined in attempts to optimize production of any required metabolite.

This isolate produced three different structural classes of natural products – the pyrrolizidines, *S*-methyl benzothioate and phenolic carboxylic derivatives. This indicates isolate LA3L2 is likely to have a wide range of biosynthetic capabilities. Thus, the One Strain-Many Compounds (OSMAC) concept of Bode *et al.* (2002) could be worthy of application in the case of *Streptomyces* sp. (LA3L2) using nutritional and environmental conditions.

Chapter 6

A Malaysian *Paecilomyces* sp. (PR10T2)

6.1 Introduction

Fungi belonging to the genus *Paecilomyces*, which is mainly an insect pathogenic group have been a source of a wide range of bioactive natural products (Isaka *et al*., 2003). Examples include paecilodepsipeptide A (antimalarial and antitumour cyclohexadepsipeptide) from *P. cinnamomeus* (Isaka *et al*., 2007), deoxymilitarinone A (neuritogenic pyridone alkaloid) from the insect pathogenic fungus *P. farinosus* (Cheng *et al.*, 2006), paecilotoxins (highly toxic linear peptides, also designated as leucinostatins) from *P. lilacinus* (Fukushima *et al*., 1983a; Fukushima *et al*., 1983b; Mikami *et al*., 1989), paeciloquinones (anthraquinones, protein tyrosine kinase inhibitors) from *P. carneus* (Frendenhagen *et al*., 1995), paecilosetin (an antibiotic tetramic acid derivative) from *P. farinosus* (Lang *et al*., 2005) and a series of trichothecanes from *P. tenuipes* (Kikuchi *et al*., 2004a; Kikuchi *et al*., 2004b; Kikuchi *et al*., 2004c). Representative secondary metabolites from marine-derived *Paecilomyces* spp., however, are quite small with only a few reported so far. These are paecilospirone, a spiroacetal compound, isolated from marine *Paecilomyces* sp. (Namikoshi *et al*., 2000), the polyketide, deoxynortrichoharzin, isolated from a sponge-derived *Paecilomyces* cf. *javanica* (Rahbaek *et al*., 1998) and sorbicillinoid urea, isolated from intertidal *P. marquandii* (Cabrera *et al*., 2006).

6.2 *Paecilomyces* sp. (PR10T2)

Isolate PR10T2 was obtained from the tissue portion of a sea fan collected in Pulau Redang Marine Park, Malaysia in September 2005 (see Figure 6.1). Stock cultures are maintained at the Fisheries Research Institute Malaysia, Pulau Pinang. The isolate was identified on the basis of colony morphology and sporulation characteristics as a *Paecilomyces* sp. by Assoc. Prof. Anthony Cole, School of Biological Sciences, University of Canterbury.

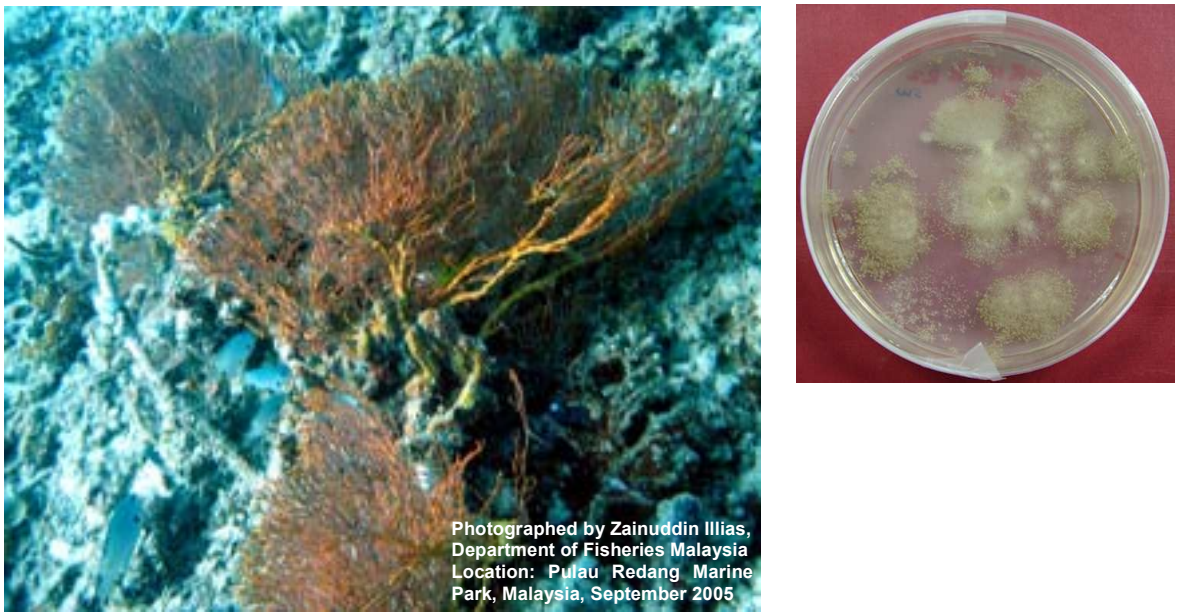


Figure 6.1: left: sea fans; right: a 7 day-culture of *Paecilomyces* sp. (PR10T2) on PYGA medium.

The extract from this isolate was further investigated because the metabolite responsible for the activity exhibited no UV/R_t correlation with known compounds when analyzed by HPLC. This chapter presents the investigation of extract F6439 using the CapNMR technique.

6.3 Investigation of small scale extract (F6439)

Extract F6439 was obtained from the small scale extraction of isolate PR10T2 (see **Chapter Two** - *Experimental*) and exhibited moderate cytotoxicity (IC_{50}12,881 ng/mL) against P388 cells, but was not active against *B. subtilis*, *P. aeruginosa* and *C. albicans* (see **Chapter Three** – *Preliminary screening*). The HPLC screening revealed that one of the compounds, NAM 6-1 eluted at 17.5 min (see Figure 6.2) coincided with the bioactivity region observed for the extract.

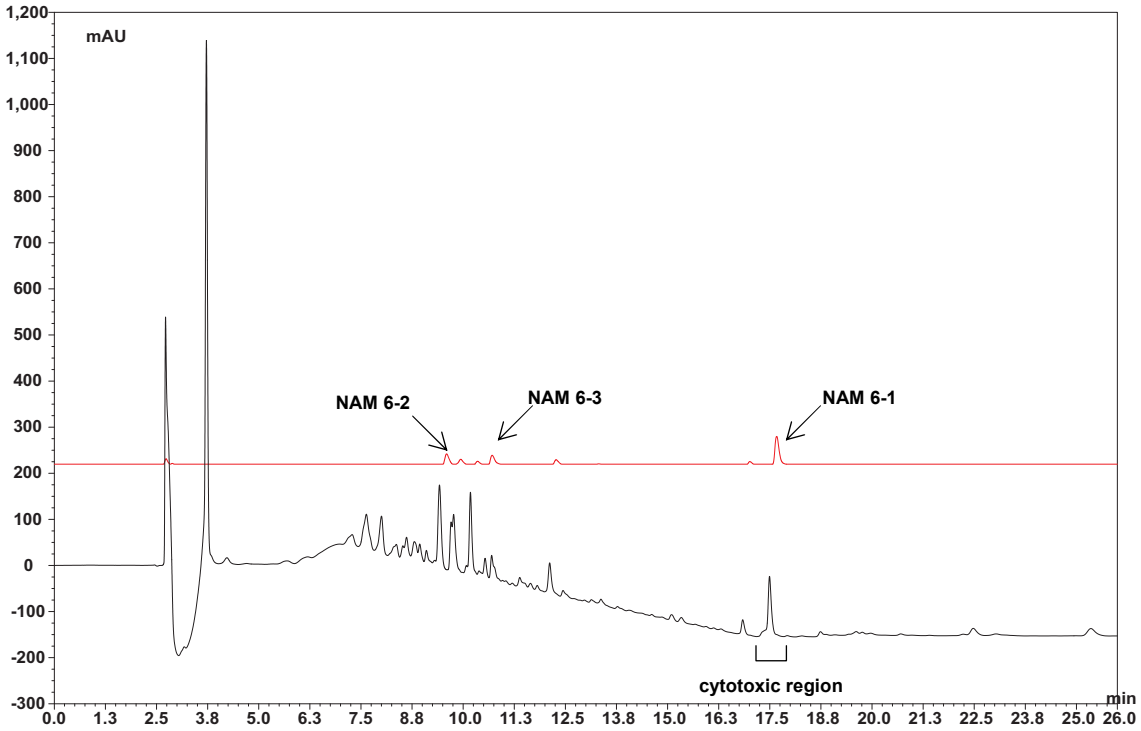


Figure 6.2: HPLC chromatogram of F6439 showing overlay of ELSD detection for compound NAM 6-1.

The UV spectrum of compound NAM 6-1 is shown in Figure 6.3, but showed no exact match to any of the compounds in the HPLC-UV/R_t library.

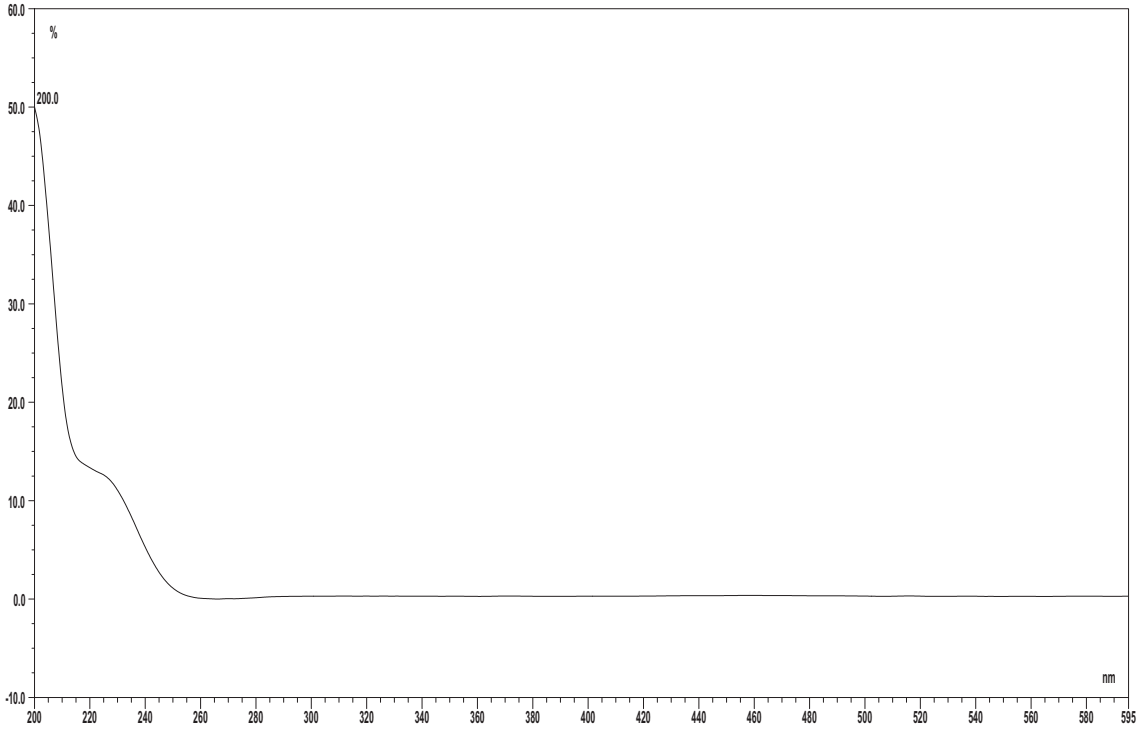


Figure 6.3: UV profile for compound NAM 6-1.

A total of 1750 µg of the crude extract (F6439) was injected on to the HPLC (by four times injections) and the compounds were collected into microtitre plates. The HPLC chromatogram of the first injection of the crude extract (1000 µg) is shown in Figure 6.4. Details of the HPLC MTT procedures are discussed in **Chapter Two** (*Experimental*). The pure compound (10 µg) was obtained from the total 1750 µg of the crude extract by combining two wells (F10 and F11) of the four microtitre plates and was analyzed using the CapNMR technique. The other minor metabolites (NAM 6-2 and NAM 6-3) were not further investigated due to insufficient amount of materials obtained.

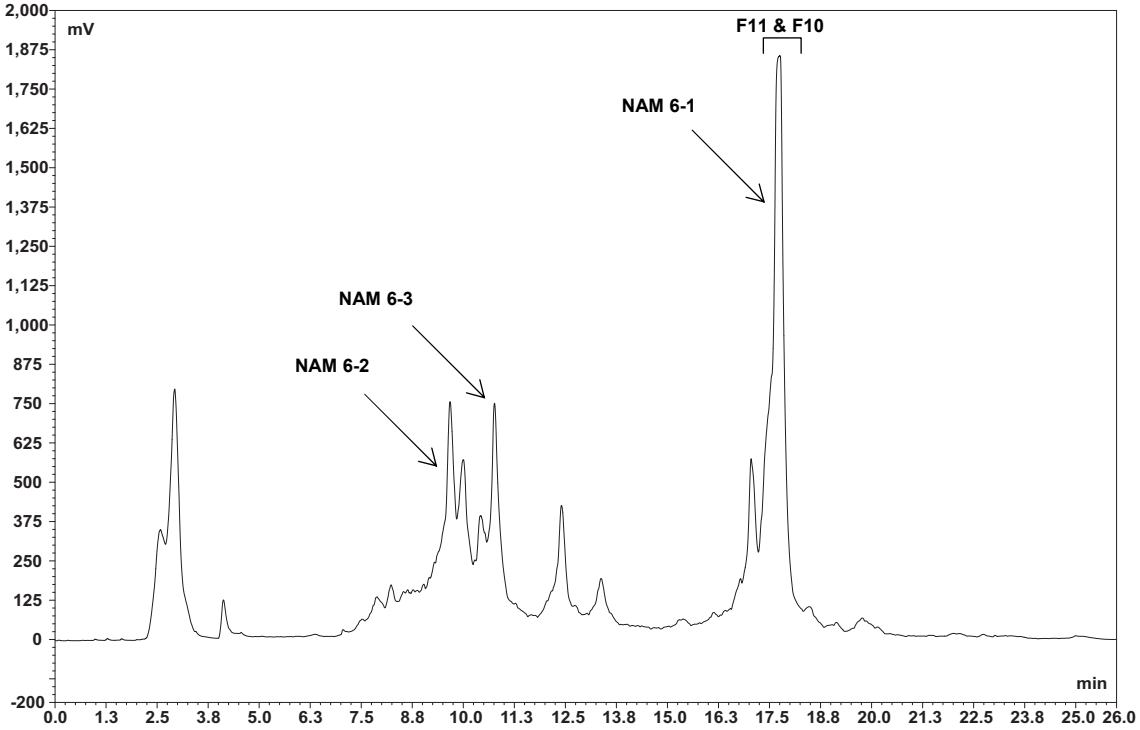


Figure 6.4: HPLC chromatogram of 1000 µg of crude extract F6439 showing the main compound NAM 6-1 collected into wells F11 and F10 of the microtitre plate.

6.4 Structural elucidation of compound NAM 6-1

The ^{1}H NMR spectrum of NAM 6-1 (see Figure 6.5) clearly showed the presence of one triplet and three doublet methyls (δ_H 0.8 – 1.1). NAM 6-1 had a molecular mass of 427 Da ($[M+H]^+$) (see Figure 6.6). A search in the AntiMarin database based on four methyl groups (three doublets and a triplet) and a molecular weight of 425 – 427 Da resulted in no match with compounds in the database. It was then noted that the molecular mass of compound NAM 6-1 ($[M+H]^+$ 427 Da) was rather too high to fit readily with the ^{1}H NMR features unless the compound was a symmetric dimer of some description.

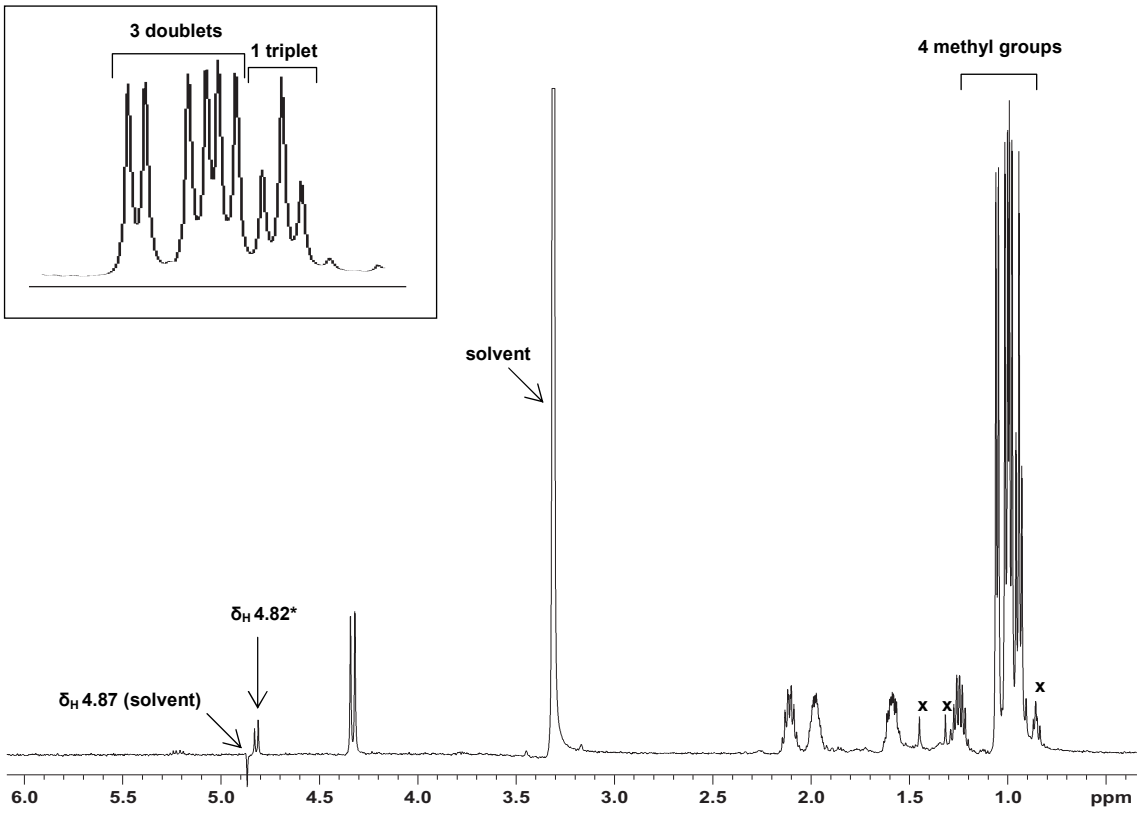


Figure 6.5: ^{1}H NMR spectrum of NAM 6-1 in CD_3OD obtained from F6439. Insert: enlargement of four methyl groups. * signal is reduced in intensity by partial saturation arising from irradiation of the HDO signal at δ_H 4.87. Crosses indicate impurities.

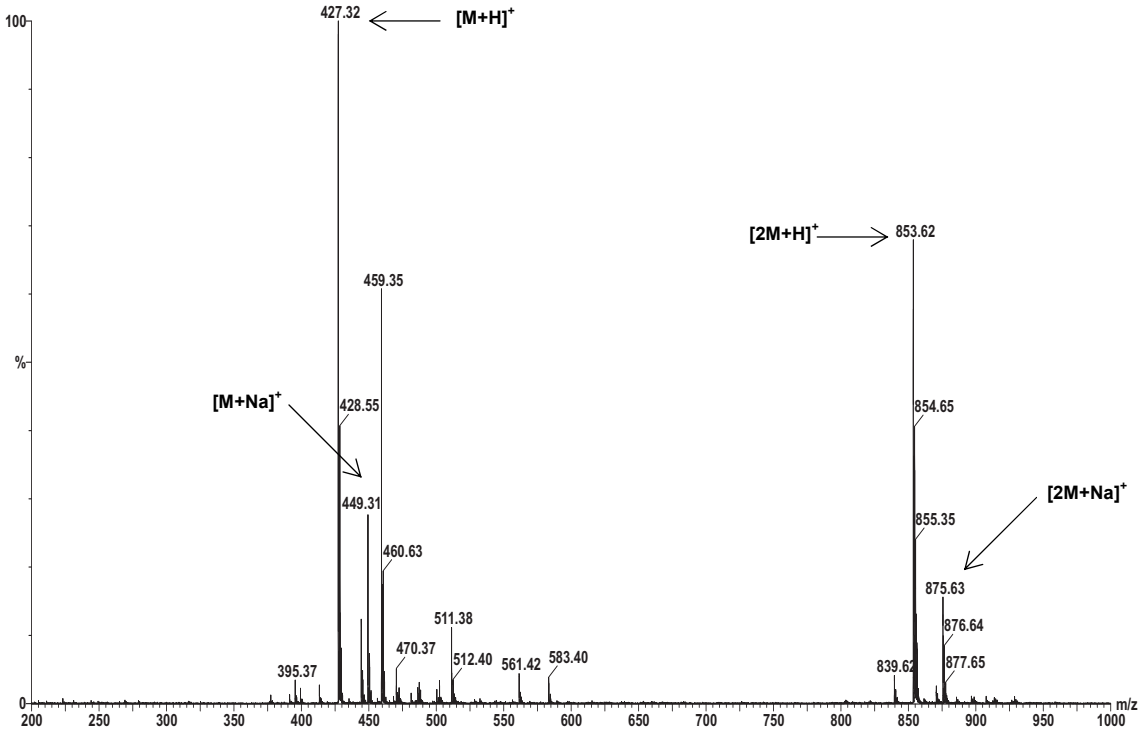


Figure 6.6: ESIMS spectrum of compound NAM 6-1.

An attempt was then made to search in the AntiMarin database for compounds with double the number of protons used in the original search that is, a search for compounds with a total of eight methyl groups (six doublets and two triplets) and molecular mass of 425 – 427 Da. This resulted in one hit (see Figure 6.7).

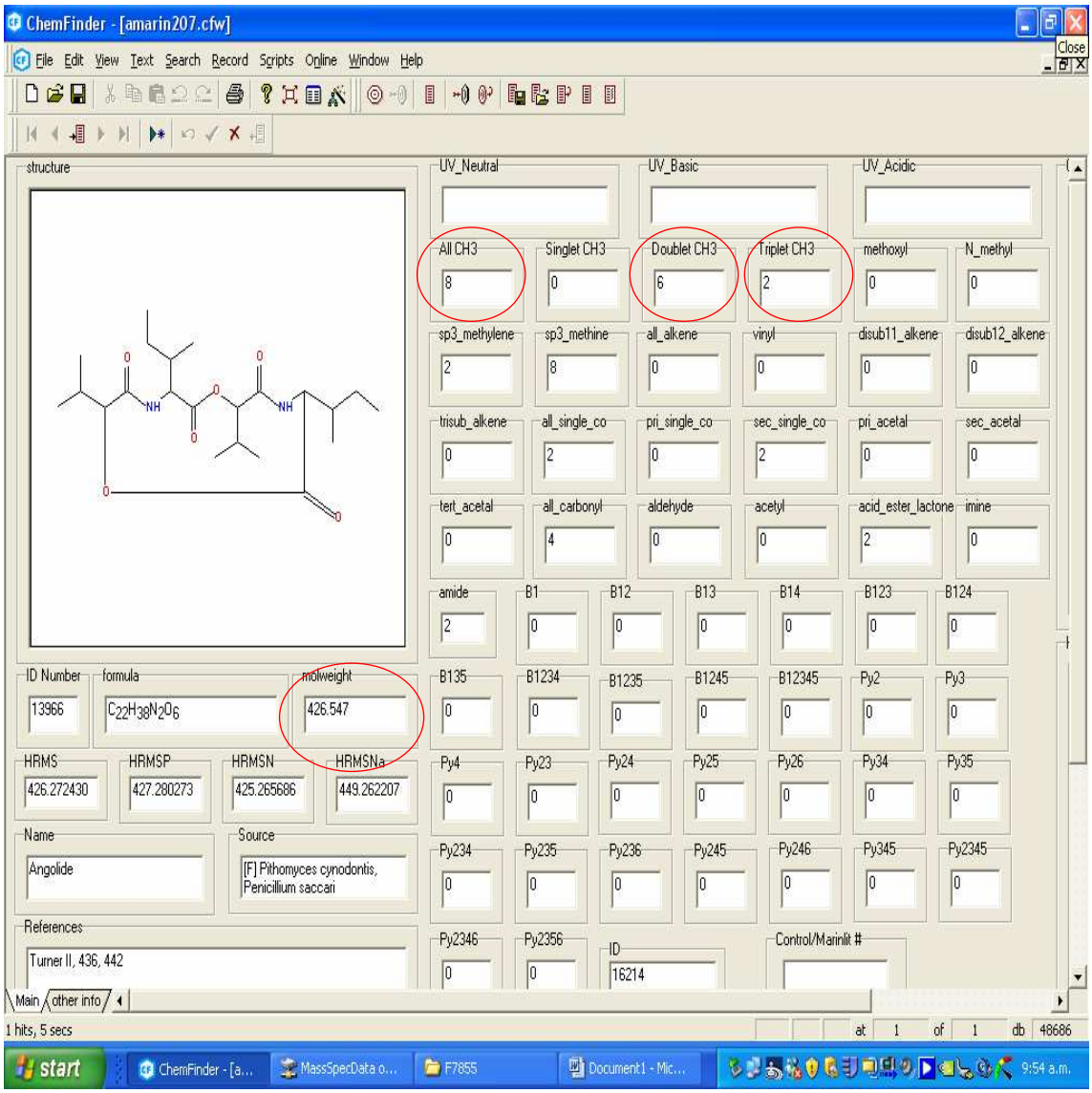
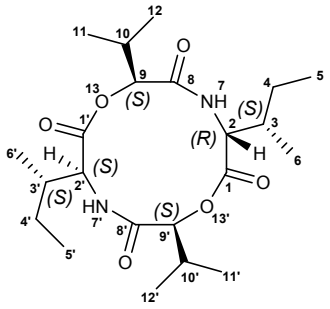


Figure 6.7: Screenshot of AntiMarin database search result for compound NAM 6-1 using the ^{1}H NMR and MS spectral data resulting in 1 hit.



(6.1)

(cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-D-*allo*-isoleucyl)

This compound was angolide (**6.1**), a cyclic depsipeptide reported from the fungi, *Pithomyces* spp. (Macdonald and Shannon, 1964; Russel, 1965; Ellis *et al.*, 1966; Russel, 1988). Angolide (**6.1**) had a molecular mass of 427 Da ($[M+H]^+$), but the structural identification had been based only on acid hydrolysis of the amino acids (Macdonald and Shannon, 1964; Russel, 1965; Ellis *et al*., 1966; Okotore and Russel, 1972; Russel, 1988).

As there had been no publication of NMR spectral data for angolide (**6.1**) to compare with the acquired NMR data of NAM 6-1, further NMR experiments were conducted. Compound NAM 6-1 was analyzed with COSY, HSQC and HMBC experiments to allow complete structural elucidation. The HSQC and HMBC spectra are shown in Figures 6.8 and 6.9, respectively.

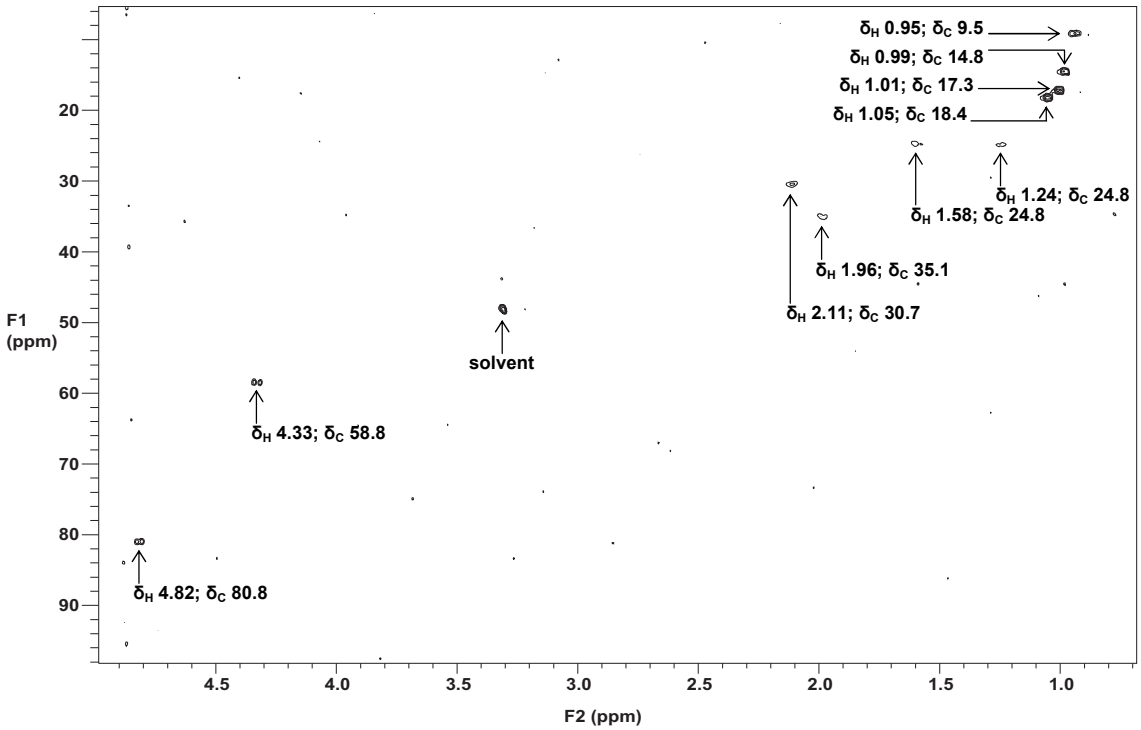


Figure 6.8: HSQC DEPT spectrum of compound NAM 6-1.

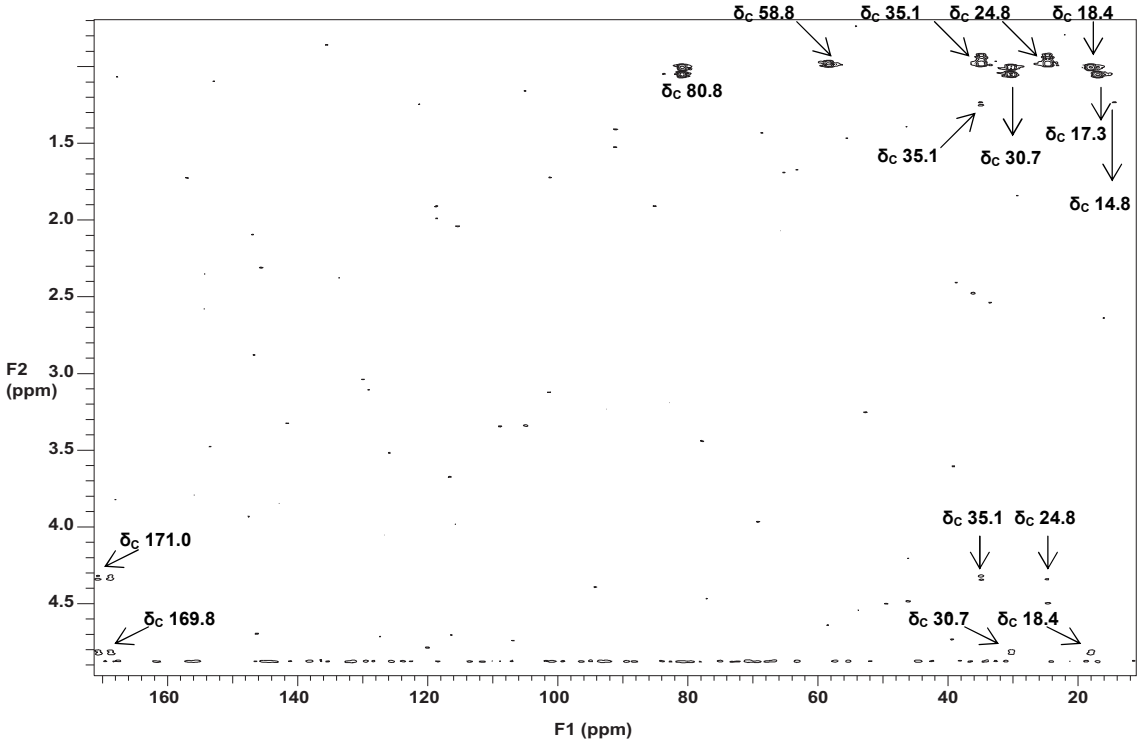


Figure 6.9: HMBC spectrum of compound NAM 6-1.

The COSY spectrum showed strong coupling between the protons at δ_H 2.11 and 4.82. The proton at δ_H 2.11 in turn was coupled to methyl protons at δ_H 1.01 and 1.05. The $^1J_{CH}$ correlation in the HSQC spectrum (Figure 6.8) showed that the protons at δ_H 1.01, 1.05 and 2.11 were attached to carbons at δ_C 17.3, 18.4 and 30.7, respectively. The proton at δ_H 4.82 was attached to a carbon at δ_C 80.8, indicated that this carbon was oxygenated. The HMBC spectrum (Figure 6.9) showed long range correlation of both the methyl protons at δ_H 1.01 and 1.05 to carbons at δ_C 30.7 (δ_H 2.11) and 80.8 (δ_H 4.82). The methyl protons (δ_H 1.01 and 1.05) and carbons (δ_C 18.4 and 17.3) were self-correlated, while the proton at δ_H 4.82 showed long range correlations with carbons at δ_C 18.4 (δ_H 1.05) and 30.7 (δ_H 2.11). Two high field carbon resonances at δ_C 169.8 and 171.0, suggesting of carbonyl groups, were correlated with the proton at δ_H 4.82 (δ_C 80.8). Based on these data, a fragment of the structure of compound NAM 6-1 was defined as illustrated in Figure 6.10. This corresponds to a valyl-type side chain.

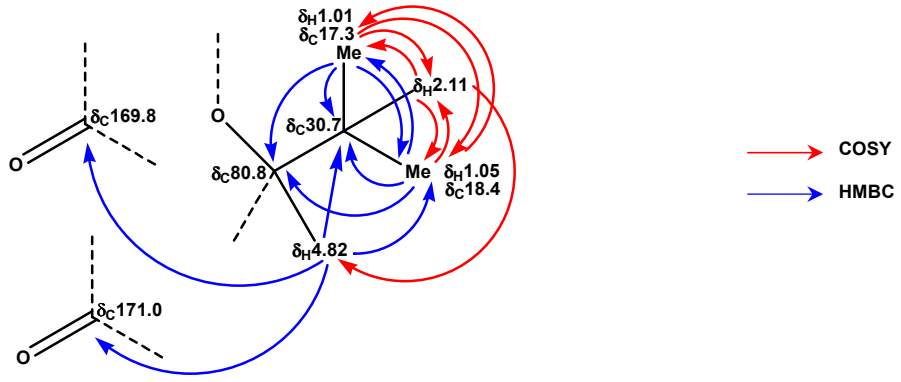


Figure 6.10: First fragment of compound NAM 6-1 deduced from COSY, HSQC and HMBC correlations.

Two protons at δ_H 1.24 and 1.58 were strongly coupled to each other and to the methyl protons (δ_H 0.95, t). The $^1J_{CH}$ correlation from the HSQC spectrum showed that the two protons at δ_H 1.24 and 1.58 were attached to the same carbon (δ_C 24.8), confirming to the coupling pattern of the triplet methyl (δ_H 0.95, δ_C 9.5). The methylene protons (δ_H 1.24 and 1.58) also showed weak coupling with the proton at δ_H 1.96 (δ_C 35.1), which in turn strongly coupled to the doublet methyl protons (δ_H 0.99) and to the proton at δ_H 4.33. The carbon assignment of this proton (δ_H 4.33, δ_C 58.8) suggested that this carbon was possibly attached to a nitrogen. In the HMBC spectrum these were long range correlation of both methyl protons (δ_H 0.95 and 0.99) to carbons at δ_C 24.8 (δ_H 1.24; 1.58) and δ_C 35.1 (δ_H 1.96). The methyl protons at δ_H 0.99 were also correlated to the carbon at δ_C 58.8 (δ_H 4.33). The proton at δ_H 1.24 showed long range correlation with carbon at δ_C 35.1 (δ_H 1.96) and a weak correlation with a carbon at δ_C 14.8 (δ_H 0.99). It was noted that the two carbon signals at δ_C 169.8 and 171.0 that were correlated with the proton at δ_H 4.82 (δ_C 80.8) previously mentioned also showed correlation with the proton at δ_H 4.33 (δ_C 58.8). Interpretation of these data suggested the other structural fragment of NAM 6-1 is an isoleucyl (see Figure 6.11).

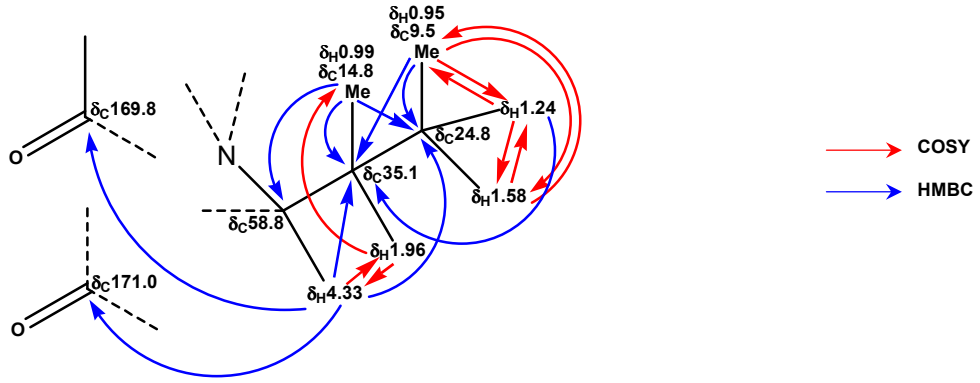


Figure 6.11: Second fragment of compound NAM 6-1 deduced from COSY, HSQC and HMBC correlations.

As both the valyl and isoleucyl-type side chains in NAM 6-1 were correlated with both carbonyl groups at δ_C 169.8 and 171.0, two peptide fragments of (-hydroxyisovaleryl-isoleucyl-) were suggested. The only linkage possible for these two fragments was to form a cyclo (-hydroxyisovaleryl-isoleucyl-hydroxyisovaleryl-isoleucyl-). The apparent symmetry of the molecule comes from the observation of only one set of methyl, methine and methylene resonances for each of the two hydroxyisovaleryl and two isoleucyl sub-structures. Confirmation of the symmetric nature came from close examination of the sets of α-protons. These protons, δ_H 4.33 and 4.82, are closer to the chiral centres than the methyl groups. Again, only two clear set of doublets were observed (see Figure 6.5). If NAM 6-1 had been asymmetric, the four protons were expected to give four sets of doublets for the protons in the NMR spectrum.

The ACD/Labs prediction software was then used to simulate the ^{1}H and ^{13}C NMR chemical shifts of angolide (**6.1**) for comparison with the experimental data obtained for compound NAM 6-1 (see Table 6.1). The comparison showed that the calculated data for angolide (**6.1**) and compound NAM 6-1 were almost identical suggesting that compound NAM 6-1 was similar to the structural features of angolide (**6.1**) as a 12-membered ring cyclic tetradepsipeptide with an ester-amide-ester-amide sequence of linkages.

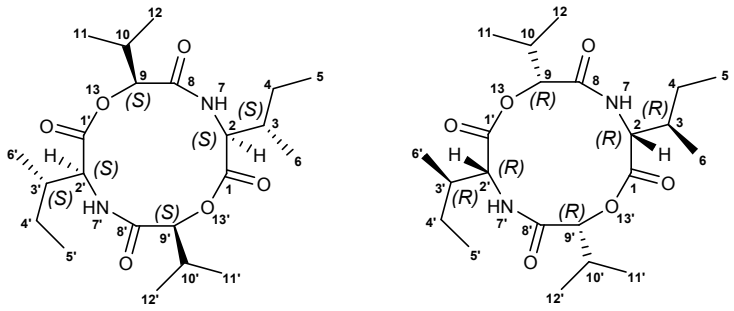
Table 6.1: Comparison of ^{1}H and ^{13}C NMR data of compound NAM 6-1 and of the simulated values of angolide (**6.1**).

Position	**NAM 6-1**[b]		**(6.1)**[a]	
	δ_H	**δ_C**	**δ_H**	**δ_C**
1		171.0		175.0
2	4.33	58.8	3.99	57.6
3	1.96	35.1	1.91	36.1
	-	-	-	-
4	1.24	24.8	1.41	27.1
	1.58		1.65	
5	0.99	14.8	0.95	15.6
6	0.95	9.5	1.12	11.6
7 (-NH-)			5.41	
8		169.7		169.6
9	4.82	80.8	5.19	77.2
10	2.11	30.7	2.36	34.0
11	1.01	17.3	0.95	17.4
12	1.05	18.4	0.96	19.0
13 (-O-)				

[a] These data were simulated by ACD/Labs software, Version 10; [b] These data were recorded at 500 MHz in CD_3OD.

A question, however arose regarding the structural configuration of NAM 6-1 to those of angolide (**6.1**). The reported angolide (**6.1**) contained a mixture of L and D-amino acids (two L-α-hydroxyisovaleryl, one L-isoleucine and one D-*allo*-isoleucine) and its structure (**6.1**) (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-D-*allo*-isoleucyl) is asymmetric. NAM 6-1 on the other hand, was deduced based on the NMR spectral data as symmetric.

A symmetrical form of NAM 6-1 must accommodate a homoisomeric configuration of the peptide fragments. The reasonable structures for NAM 6-1 that could be drawn based on homoisomeric configurations were (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl) (or its enantiomer) (see Figure 6.12) which would have a two-fold axis of symmetry, or (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-D-α-hydroxyisovaleryl-D-isoleucyl) (see Figure 6.13) with a centre of inversion. The ^{1}H NMR data alone would not distinguish another possible diastereoisomer, also with a centre of inversion, (cyclo-L-α-hydroxyisovaleryl-D-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl) (see Figure 6.14). These possible configurations of NAM 6-1 therefore did not fit with the reported angolide (**6.1**), and NAM 6-1 must therefore be considered as a new compound.

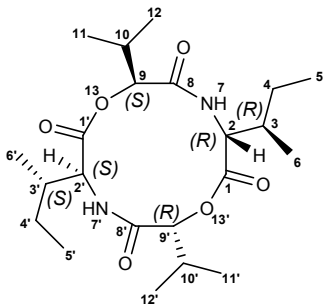


(a) **(b)**

a: (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl)

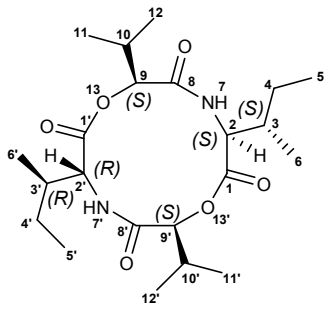
b: (cyclo-D-α-hydroxyisovaleryl-D-isoleucyl-D-α-hydroxyisovaleryl-D-isoleucyl)

Figure 6.12: Homoisomeric configurations of NAM 6-1: (a) (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl) and (b) its enantiomer.



(cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-D-α-hydroxyisovaleryl-D-isoleucyl)

Figure 6.13: Homoisomeric configuration of NAM 6-1: (cyclo-L-α-hydroxyisovaleryl-L-isoleucyl-D-α-hydroxyisovaleryl-D-*allo*-isoleucyl).



(cyclo-L-α-hydroxyisovaleryl-D-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl)

Figure 6.14: Homoisomeric configuration of NAM 6-1: (cyclo-L-α-hydroxyisovaleryl-D-*allo*-isoleucyl-L-α-hydroxyisovaleryl-L-isoleucyl).

6.5 Discussion

Cyclic depsipeptides are group of metabolites characterized by the occurrence of at least one ester linkage (Faulkner, 1994; Burja *et al*., 2001; Faulkner, 2002). They are of great interest because they possess a diverse range of bioactivity including antitumour, antibiotic, antifungal, immunosuppressant and anti-inflammatory activities. Various types of cyclodepsipeptides have been reported from marine-derived fungi such as the spicellamides A and B from *Spicellum roseum* (Kralj *et al*., 2007), zygosporamide from *Zygosporium masonii* (Oh *et al*., 2006), guangomides A and B from an unidentified sponge-derived fungus (Amagata *et al*., 2006b), IB-01212 from *Clonostachys* sp. (Cruz *et al*., 2006), aspergillicins A – E from marine sediment-derived *Aspergillus carneus* (Capon *et al*., 2003), N-methylsansalvamide A from algal-derived *Fusarium* sp. (Cueto *et al*., 2000), sansalvamide from a sea grass-derived *Fusarium* sp. (Belofsky *et al*., 1999) and exumolides A and B cyclic hexadepsipeptides from a marine plant-derived *Scytalidium* sp. (Jenkins *et al*., 1998).

Cyclic depsipeptides have not been reported from marine-derived *Paecilomyces* spp. previously. Two compounds, however, have been reported from terrestrial counterparts. The cyclic hexadepsipeptide, beauvericin has been isolated from *P. fumosoroseus* and *P. tenuipes* (Klaric and Pepeljnjak, 2005). An antimalarial and antitumour cyclohexadepsipeptide, paecilodepsipeptide A, was recently reported from *P. cinnamomeus* (Isaka *et al*., 2007). Marine invertebrates are known to be sources of structurally bioactive cyclic depsipeptides. Some noteworthy examples include the antitumour metabolites, arenastatin A from the Okinawan sponge, *Dysidea arenaria* (Kobayashi *et al*., 1994a; Kobayashi *et al*., 1994b), didemnins from a Caribbean tunicate, *Tridemnum solidum* (Rinehart *et al*., 1981), jasplakinolide from the Indo-Pacific sponge, *Jaspis* cf. *Johnstoni* (Crews *et al*., 1986) and geodiamolides from the sponge, *Geodia* sp. (Chan *et al*., 1987).

Production of cyclic depsipeptides has been reported for microorganisms associated with marine invertebrates. Examples are the dolastatins from the Western Indian Ocean sea hare, *Dolabella auricularia* (Pettit, 1997) and from the cyanophyte *Symploca* sp. (Luesch *et al*., 2001) and kahalalide from the mollusc, *Elysia rufescens* and its dietary alga, *Bryopsis* sp. (Bonnard *et al*., 2003).

The source of *Paecilomyces* sp. (PR10T2) was Pulau Redang Marine Park Malaysia which is surrounded by the South China Sea, a part of the China Sea. The China Sea (consisting of China Bo Sea, China Yellow Sea, East China Sea and South China Sea) (Zhang *et al*., 2003a) is regarded of showing great potential as a source of marine natural products (see Figure 6.16). The Chinese terrestrial waters of South China Sea have received some attention (Lin *et al*., 2000; Yang *et al*., 2002; Yin *et al.*, 2005; Yang *et al*., 2006) for natural product discovery from marine microorganisms, however, the Malaysian zone is virtually unexplored, with only metabolites from several species of the red algae *Laurencia* (Vairappan *et al*., 2001; Vairappan, 2003; Vairappan *et al*., 2004), a sponge (Fernandez *et al.*, 1992) and a crinoid (Shao *et al*., 2007) reported in the international literature.

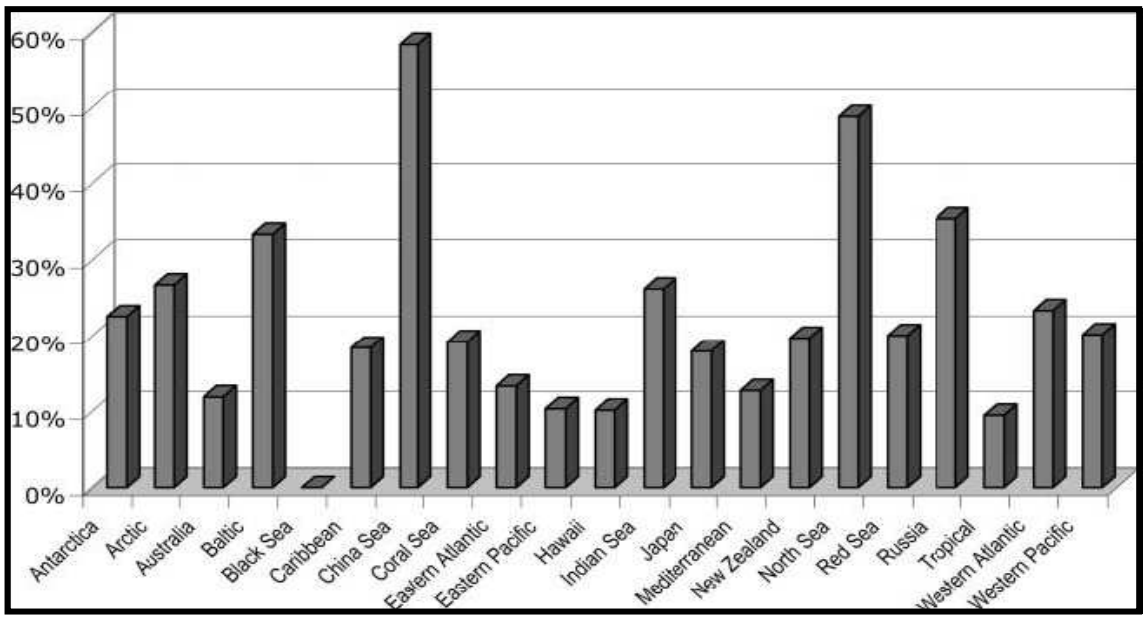


Figure 6.16: Citations for source regions for the period 2001–2005 as a percentage of the totals for 1965–2005 (from Blunt *et al*., 2007).

In this study, the amount of NAM 6-1 used for characterization (approximately 10 μg), while sufficient for establishing the structure by 1D and 2D NMR spectroscopy was insufficient to allow chemical analysis and establish the relative and absolute stereochemistry. Further attempts will be carried out to culture the producing isolate to obtain a greater amount of NAM 6-1 for this final definition work on the stereochemistry.

Chapter 7

Application of rapid techniques for chemical characterization of metabolites

7.1 Introduction

Rapid identification of natural products is an important step to distinguish between known and unknown compounds and consequently allowing the exclusion of known compounds at an earlier stage. Over the years, numerous approaches have been undertaken including chromatography-based approaches combined with MS or NMR spectroscopy and the establishment of UV spectra libraries and commercial databases.

NMR spectroscopy is probably the most versatile technique for the determination of molecular structures. The NMR technique has become increasingly more important in natural products studies with the rapid development and diversification of NMR spectroscopy. In natural products studies many of the bioactive metabolites produced are of small amount and often in an amount lower than required to carry out complete structural elucidation. With recent development of new probe technologies, the utilization of NMR as a tool for rapid characterization of metabolites has become increasingly more important.

One advancement was the production of a CapNMR - designed to allow NMR experiments by using amounts of materials much less than required for a conventional probe. Normal data acquisition using a NMR regular probe requires at least 1.0 – 2.0 mg of pure material. In the case of simple small molecules, 1D NMR coupled with MS may be enough to characterize the structure of a compound. Most natural products, however, are not small molecules and certainly not simple, therefore 2D NMR techniques are required. Among the most commonly used are HSQC and HMBC experiments. In these cases, greater quantities of pure metabolite are required, depending somewhat on the molecular structure of the compound, for an adequate 2D NMR spectrum to be achieved. The sensitivity of the capillary-scale NMR probe to enhance structural elucidation by using only small amount of natural product has been described for the plants, *Penstemon centranthifolius* (Hu *et al*., 2005) and firefly, *Lucidota atra* (Gronquist *et al*., 2005).

The CapNMR technique is currently utilized by the Marine Chemistry Group in conjunction with the AntiMarin database with MS and UV data as a tool to enhance dereplication as well as to obtain a rapid NMR data acquisition for characterization of new metabolites by using 2 – 50 μg of purified material. Less than <50 μg sample is required to perform both 1D proton and 2D homonuclear NMR experiments. The NMR features such as methyl groups and aromatic spin systems that can usually be recognized from the 1D spectrum, along with MS and UV data can make the search profile in the AntiMarin database highly discriminating as the database contains NMR data besides other various molecular descriptors such as MS and UV. For a more complex structure, about 30 – 50 μg of sample are needed to acquire 2D heteronuclear spectra such as HMBC or HSQC.

Prior to the CapNMR technique, the group's approach to rapid dereplication utilized the LC-MS-UV technique in combination with the AntiMarin database. This technique allows the dereplication of mixtures of natural products and requires only a small amount of the crude extract (1 mg/mL) and operating in conjunction with HPLC time-of-flight mass spectrometry (TOFMS) analysis. The utilization of LC combined with MS has been developed to detect the presence of known secondary metabolites from microorganisms (Filtenbort *et al*., 1983; Frisvad *et al*., 1989; Smedsgaard and Frisvad, 1996; Higgs *et al*., 2001). Another technique that combined UV spectra with LC-MS (LC-MS-UV) was used to develop a database of 474 mycotoxin and fungal metabolites (Nielsen and Smedsgaard, 2003). This database is very useful as a dereplication tool for many metabolites originating from fungi such as *Penicillium* spp. and *Aspergillus* spp. With this technique, the UV spectrum was still a valuable tool for rapid identification for metabolites with characteristic UV chromophores. However, the limitation within this technique lies with the fact that not all metabolites have characteristic UV spectra and in some cases of isomers with similar molecular formula, the UV spectra are identical.

Work of the University of Canterbury Marine Chemistry group is presently focused on fungal metabolites and an in-house HPLC-UV/R_t library database for known fungal metabolites by using the Chromeleon software on the Dionex analytical HPLC has been established. The dereplication of fungal metabolites using the library database is carried out by establishing whether any of the significant peaks showed matches (both UV chromophore and R_t) with any known compounds already present within the database. With the current rapid techniques being applied by the group, more known compounds will be inserted progressively into the library database thus enhancing it use for future dereplication.

The application of 1D and 2D CapNMR techniques for structural elucidation of metabolites was presented in **Chapter Five** (*Secondary metabolites from marine-derived Streptomyces sp. (LA3L2)*) and **Chapter Six** (*A Malaysian Paecilomyces sp. (PR10T2)*). This chapter presents an overview of the application of the CapNMR and LC-MS-UV techniques for chemical characterization of metabolites in selected bioactive extracts obtained from marine-derived actinomycetes and fungi. Characterization of known structural features by using <10 µg pure or partially pure samples is described by using the CapNMR technique. The reliability of LC-MS/UV technique to dereplicate mixtures of natural products in selected extracts is also demonstrated. The utilization of the HPLC-UV/R_t library database is briefly mentioned.

7.2 Dereplication using LC-MS-UV

Five bioactive extracts that contain significant peak(s) that were not readily dereplicated using the HPLC-UV/R_t library database are further investigated using the LC-MS-UV technique. These extracts were F5956 from a *Streptomyces* sp. (**Section 7.2.1**) and four *Penicillium* spp. extracts namely F5975 (**Section 7.2.2**), F5890 (**Section 7.2.3**), F5350 (**Section 7.2.4**) and F5868 (**Section 7.2.5**). Details of the procedures were discussed in **Chapter Two** (*Experimental*).

7.2.1 Extract F5956

Extract F5956 was obtained from a SCA culture medium of *Streptomyces* sp. (LA3L1). This small-scale extract showed good cytotoxicity (IC_{50}<97.5 ng/mL) and was active against *B. subtilis* (101% inhibition). The HPLC screening of F5956 revealed three main peaks, NAM 7-1, NAM 7-2 and NAM 7-3 appearing over the bioactive region (R_t 14.0 – 17.0 min) as illustrated in Figure 7.1. These three peaks were shown to have virtually identical UV chromophores, suggesting that they were closely related in structure (Figure 7.2).

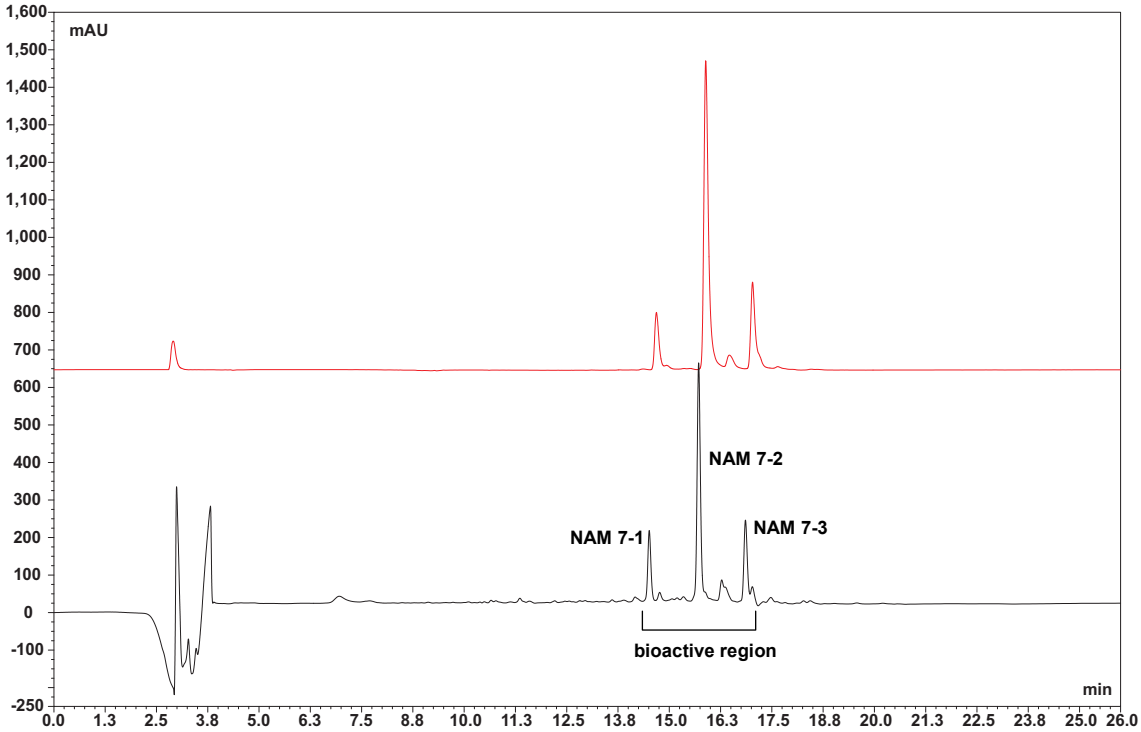


Figure 7.1: HPLC chromatogram of F5956 showing overlay of ELSD detection (top) for compounds NAM 7-1, NAM 7-2 and NAM 7-3 eluted over the bioactive region.

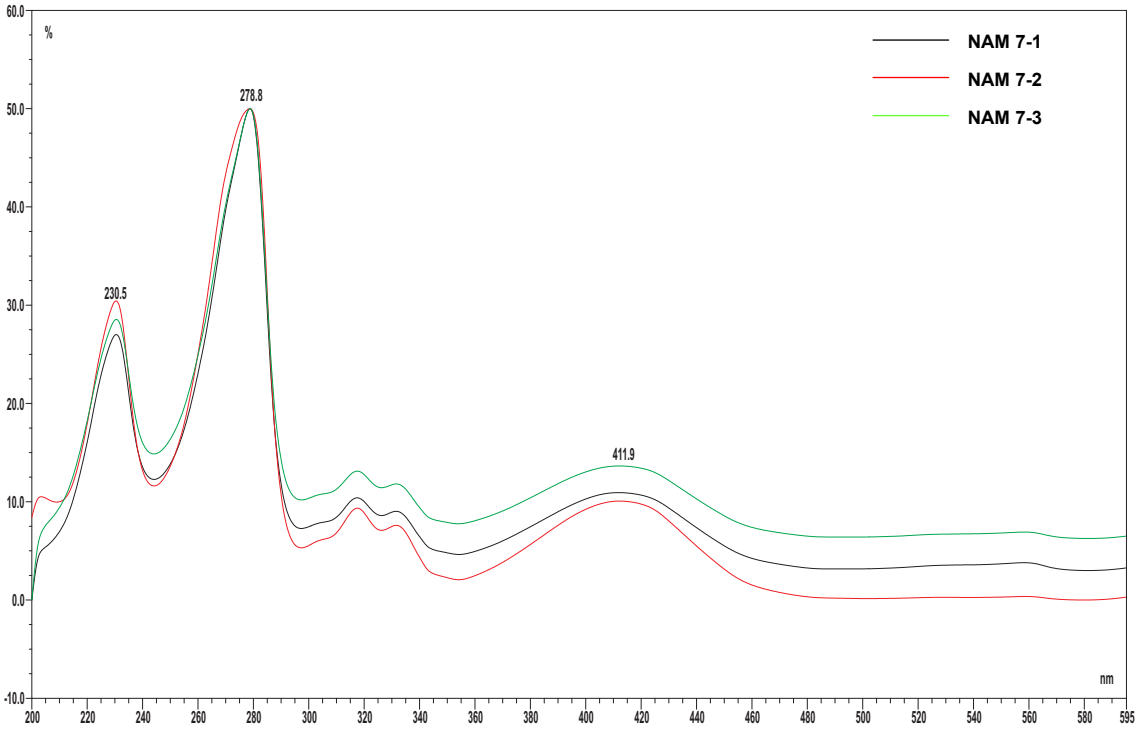


Figure 7.2: UV profile overlay for NAM 7-1, NAM 7-2 and NAM 7-3 of F5956.

The extract was subjected to HPLC TOFMS (negative ionization) analysis to further investigate compounds NAM 7-1, NAM 7-2 and NAM 7-3. The desired compounds (NAM 7-1, NAM 7-2 and NAM 7-3) were determined by correlating the total ion current (TIC) traces of the HPLC TOFMS spectrum with the UV profiles of the HPLC chromatogram (see Figure 7.3). The MS of compounds NAM 7-1, NAM 7-2 and NAM 7-3 were then determined from the corresponding TIC traces.

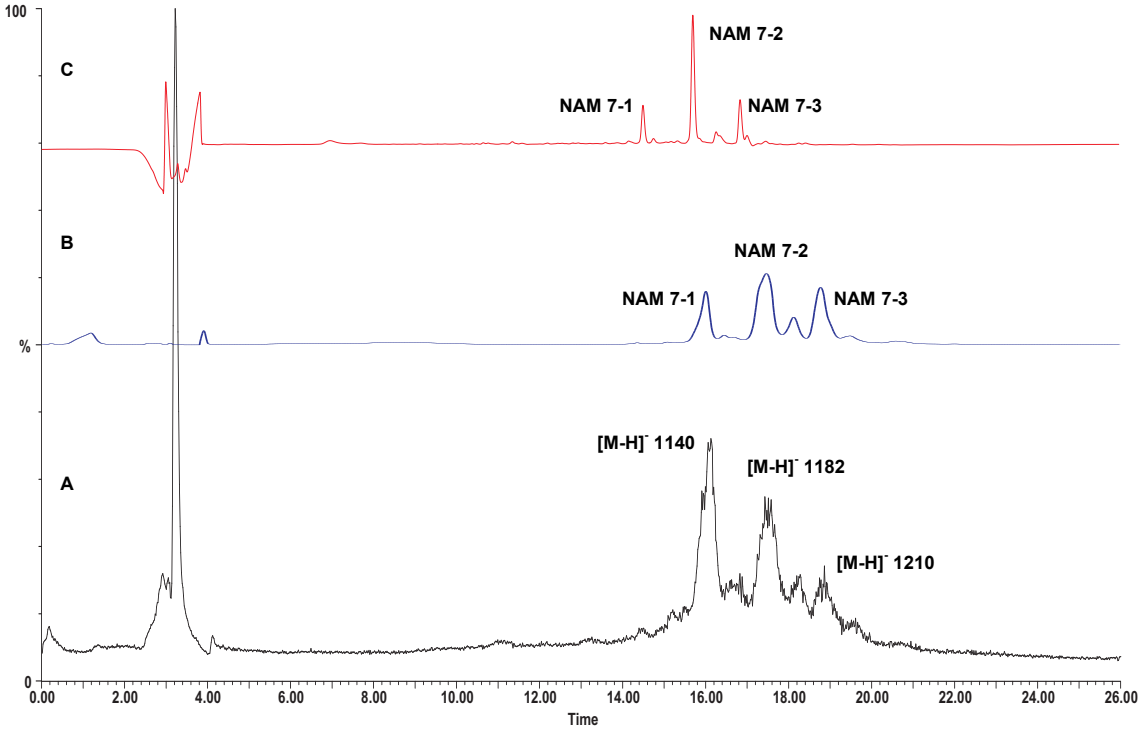


Figure 7.3: TIC chromatogram (negative) of F5956. A: TIC traces of TOFMS; B: overlay of UV profile from the corresponded TIC traces; C: overlay of corresponded HPLC-UV traces.

The results obtained for compound NAM 7-3 are discussed first in order to facilitate the discussion on the dereplication of compounds NAM 7-1 and NAM 7-2. Compound NAM 7-3 had the molecular mass of 1210 Da ($[M-H]^-$) (see Figure 7.4).

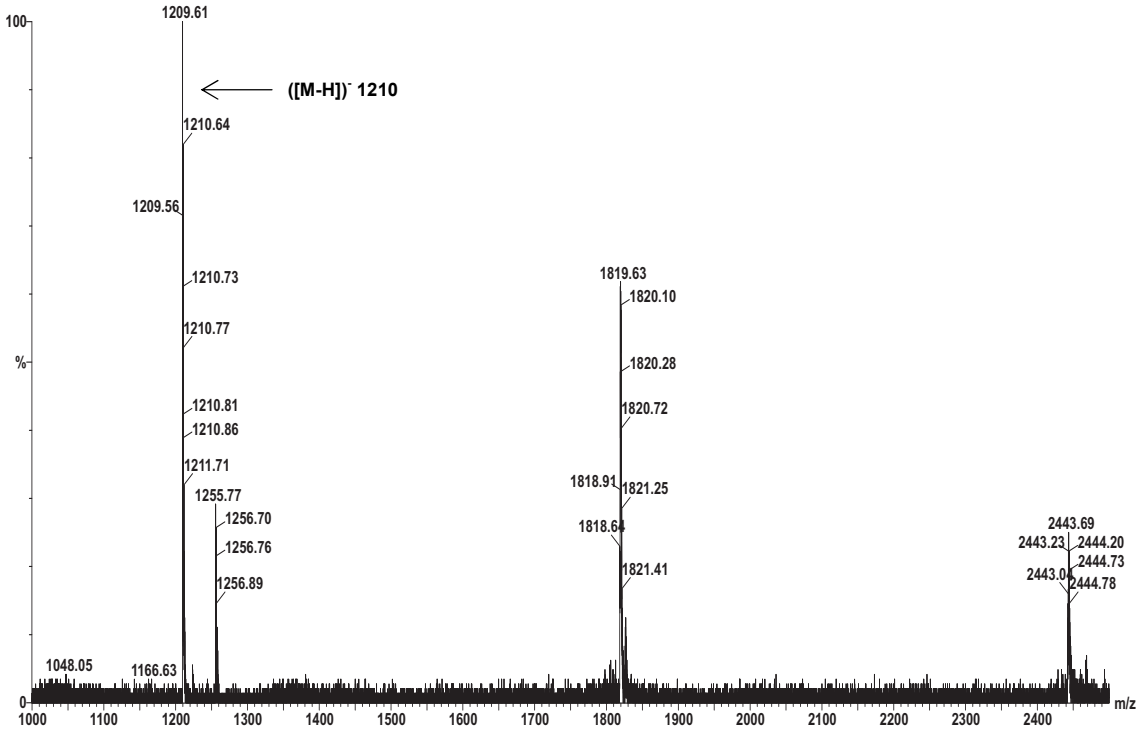
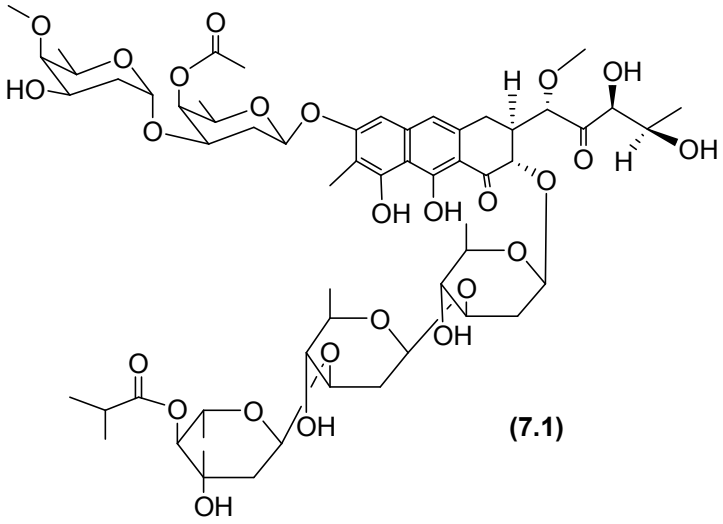
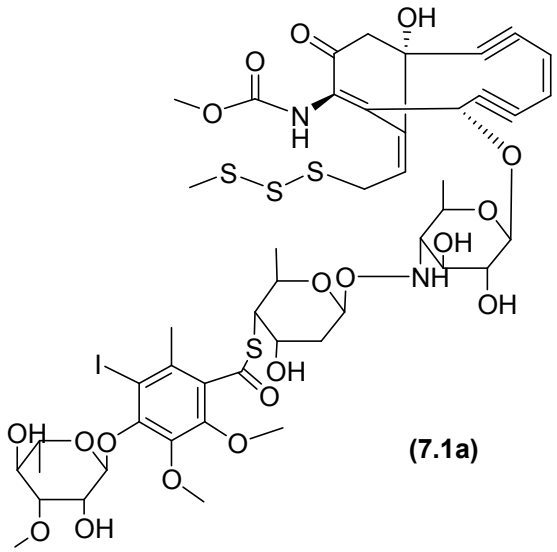


Figure 7.4: MS spectrum obtained from TIC traces of TOFMS of compound NAM 7-3 showing the ($[M-H]^-$) of 1210 Da.

Only two known compounds with molecular mass of 1210 - 1212 Da showed matches with compound NAM 7-3 in the AntiMarin database and these were chromomycin A2 (**7.1**) and calichemicin a3l (**7.1a**). The UV spectrum of compound NAM 7-3 was compared to those of the two known compounds. It showed that the UV spectrum of compound NAM 7-3 was consistent with that reported for chromomycin A2 (**7.1**) (Miyamoto *et al.*, 1967; Yoshimura *et al*., 1988), leading to the assignment of NAM 7-3 as chromomycin A2 (**7.1**).

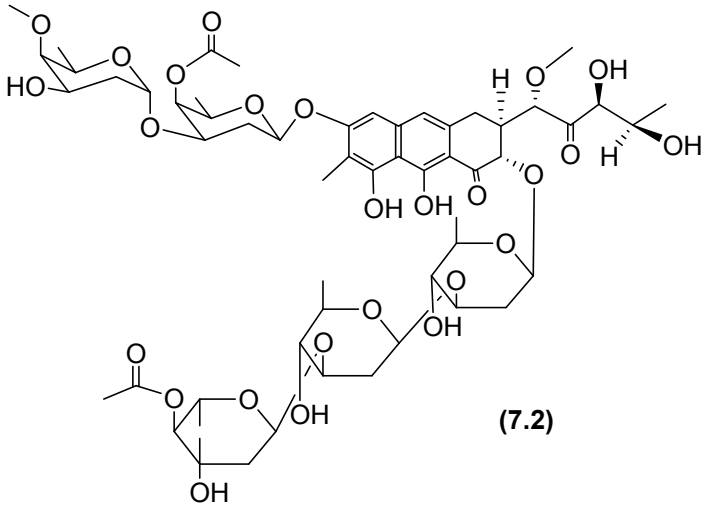


(7.1)



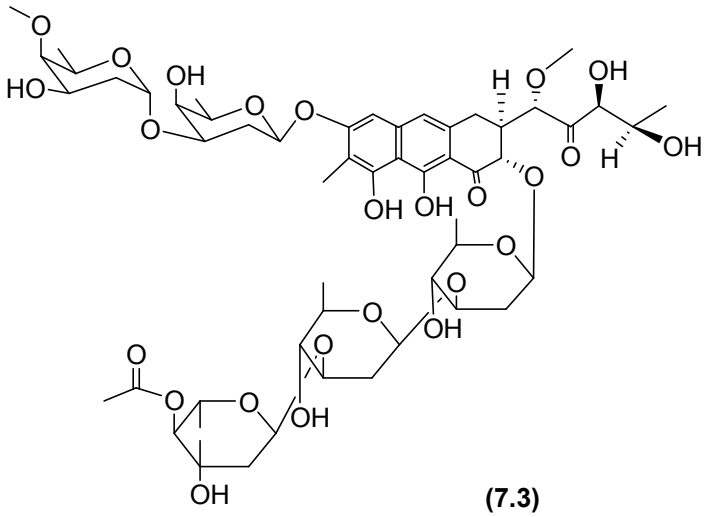
(7.1a)

Based on the similarity of their UV chromophores, NAM 7-3 was closely related in structure to compounds NAM 7-1 and NAM 7-2. The structural features of the chromomycin group of compounds were then used as a basis to identify compounds NAM 7-1 and NAM 7-2. Compound NAM 7-2 has a molecular mass of 1182 Da ([M-H]$^-$). A search in the AntiMarin database showed 15 matches with known compounds with molecular mass of 1182 – 1184 Da. Of 15 hits, only one was related to chromomycin and this was chromomycin A3 (**7.2**). The UV spectral data of compound NAM 7-2 was consistent with that reported for chromomycin A3 (Miyamoto *et al.*, 1967; Yoshimura *et al*., 1988), and was thus identified as chromomycin A3 (**7.2**).



(7.2)

Compound NAM 7-1 has a molecular mass of 1140 Da ($[M-H]^-$). A search of AntiMarin database based on the molecular masses of 1140 – 1142 Da resulted in 34 hits, of which only one correlated with the chromomycins named chromomycin 02-3D (**7.3**). The UV spectral data of compound NAM 7-1 was consistent with that for chromomycin 02-3D (**7.3**), which was a mono-deacetyl chromomycin reported by Kawano *et al*. (1990). Based on this, compound NAM 7-1 was thus identified as chromomycin 02-3D (**7.3**).



(7.3)

7.2.2 Extract F5975

Extract F5975 was obtained from a PYGA culture medium of *Penicillium* sp. (LY1L5). This small-scale extract showed fair cytotoxicity (IC_{50}5,117 ng/mL) but was inactive in the antimicrobial assays. The HPLC screening of F5975 showed the presence of two significant peaks, NAM 7-4 and NAM 7-5 eluted at 16.2 and 17.6 min, respectively (Figure 7.5). The HPLC MTT plate assay showed only compound NAM 7-5 was responsible for the activity.

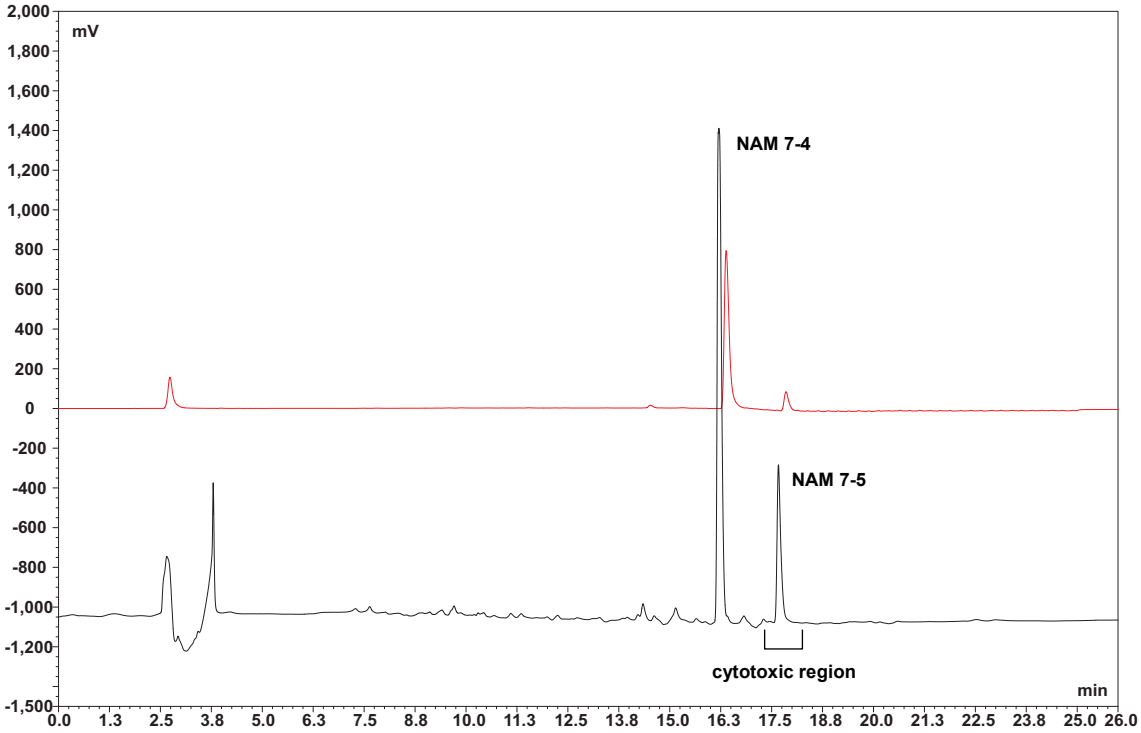


Figure 7.5: HPLC chromatogram of F5975 showing overlay of ELSD detection (top) for compounds NAM 7-4 and NAM 7-5.

The UV spectra of compounds NAM 7-4 and NAM 7-5 are shown in Figure 7.6. Compound NAM 7-4 showed a match with the known compound, cycloaspeptide A (**7.4**) from the HPLC-UV/R_t library database, while no exact matches were found for compound NAM 7-5.

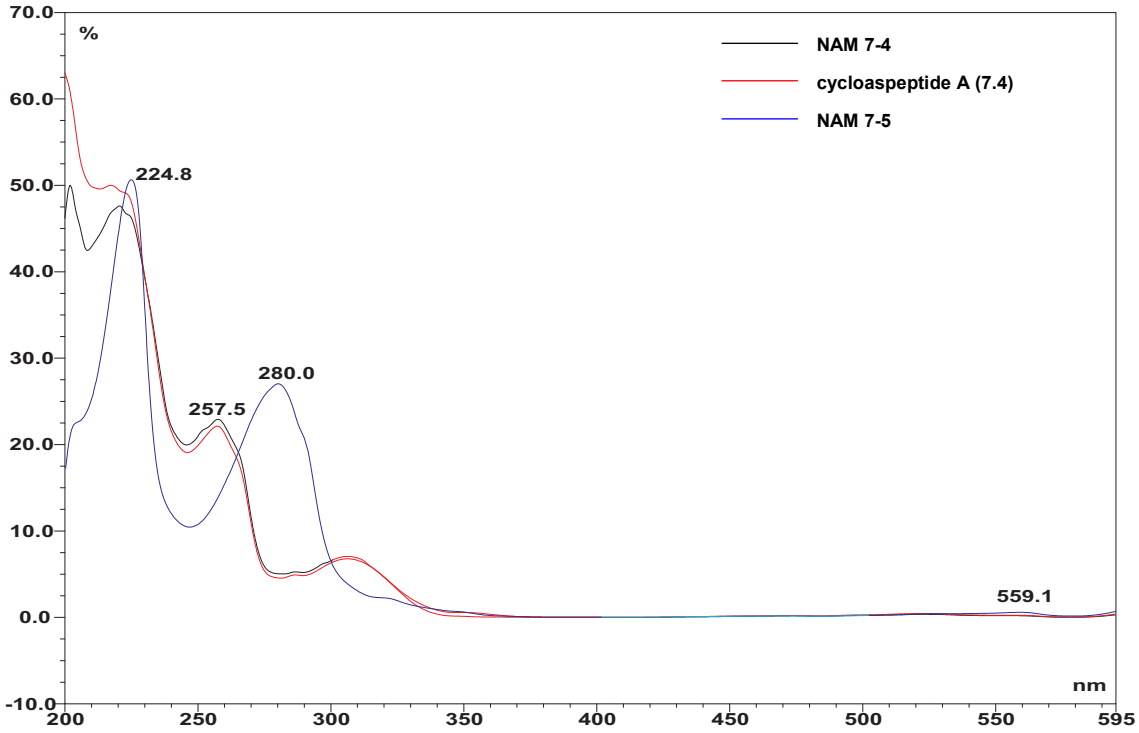
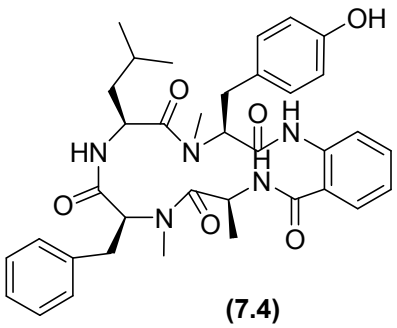


Figure 7.6: UV profile of F5975 showing overlay of compound NAM 7-4 (black) and the UV library hit (red) with the known cycloaspeptide A (**7.4**) in the HPLC-UV/R_t library database. An overlay of the UV spectrum of compound NAM 7-5 is shown in blue.



(7.4)

Compound NAM 7-5 was analyzed by HPLC TOFMS and MS spectrum is shown in Figure 7.7. The MS spectrum showed the presence of a dimeric cluster ion $[2M+H]^+$ that assigned the $[M+H]^+$ ion as 337. A search in the AntiMarin database on compounds with molecular weight of 335 – 337 Da resulted in 207 hits. Further search in the published database on fungal metabolites (Nielsen and Smeedsgaard, 2003) resulted in a match with the known α-cyclopiazonic acid (**7.5**).

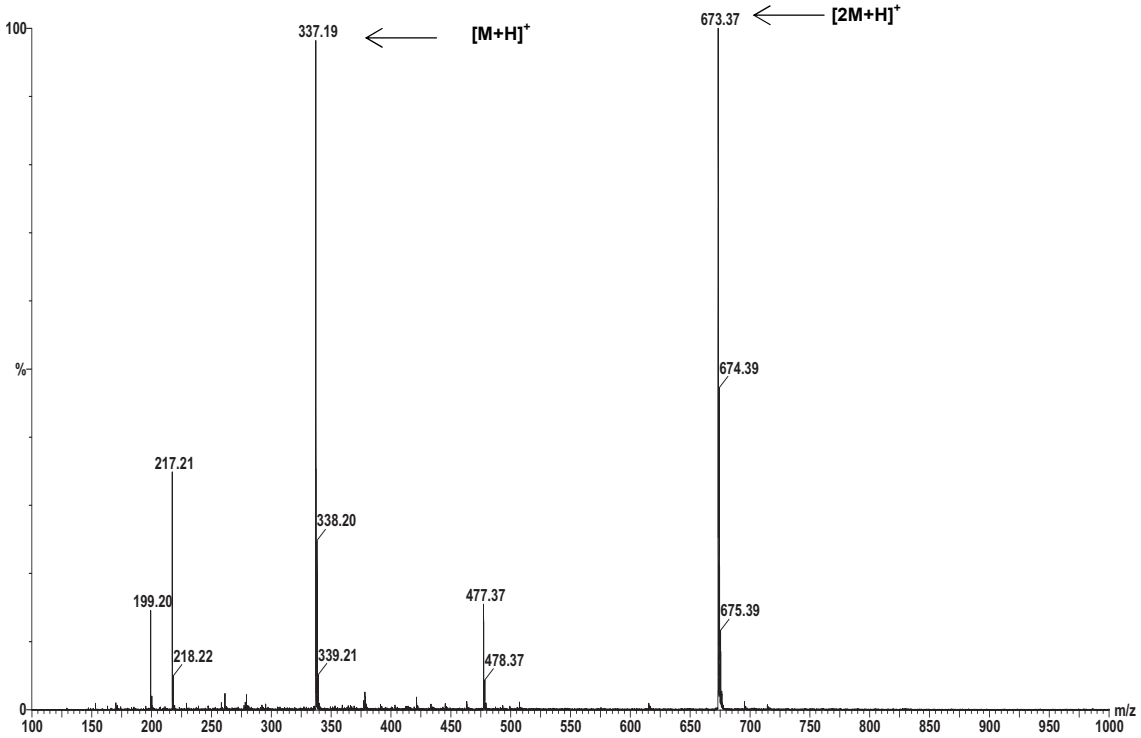
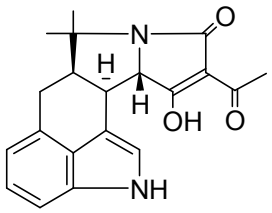


Figure 7.7: MS spectrum of compound NAM 7-5 obtained from TIC traces of F5975.



(7.5)

7.2.3 Extract F5890

Extract F5890 was obtained from a PYGA culture medium of *Penicillium* sp. (KK3T23). This small-scale extract showed fair cytotoxicity (IC_{50}3,177 ng/mL) but was inactive in the antimicrobial assays. The HPLC screening showed three main peaks NAM 7-6, NAM 7-7 and NAM 7-8 eluted over the inactive region (10.0 – 15.0 min) and a cytotoxic peak NAM 7-9 eluted at 17.7 min (see Figure 7.8). The MS of each peak was determined from TIC traces of the HPLC TOFMS spectrum (positive ionization).

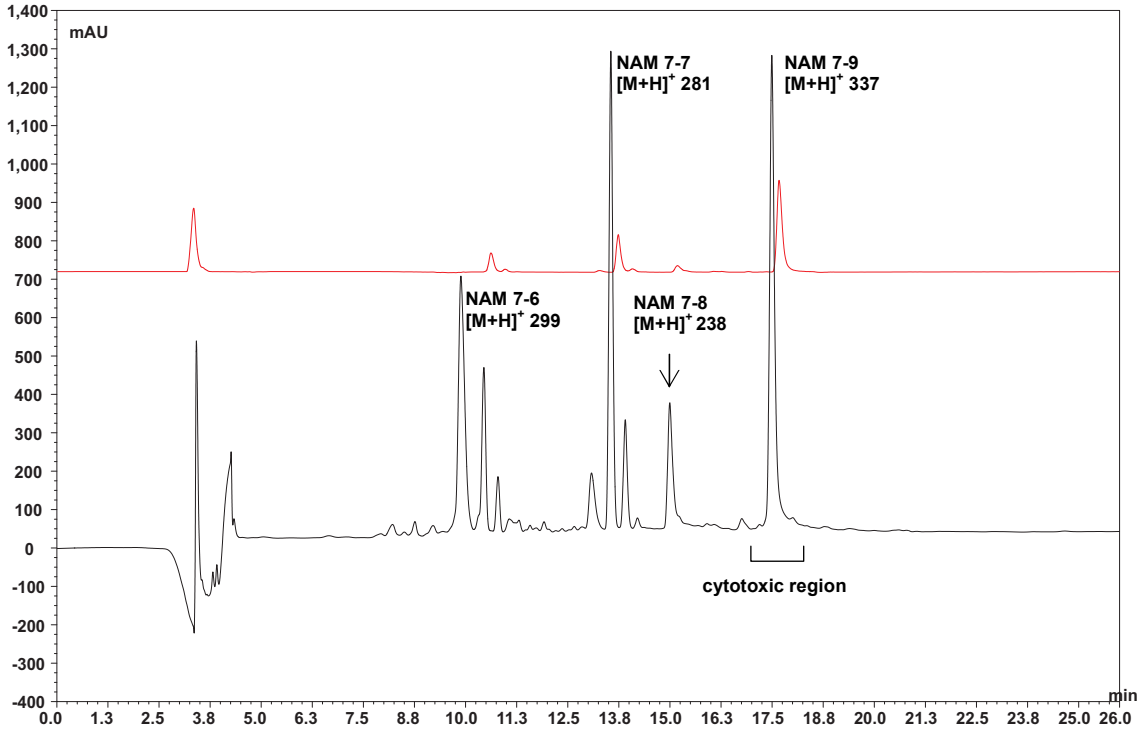


Figure 7.8: HPLC chromatogram of F5890 showing overlay of ELSD (top) for the main compounds NAM 7-6, NAM 7-7, NAM 7-8 and NAM 7-9.

Compound NAM 7-9 showed a match with the known compound, α-cyclopiazonic acid (**7.5**) from the HPLC-UV/R_t library database (Figure 7.9).

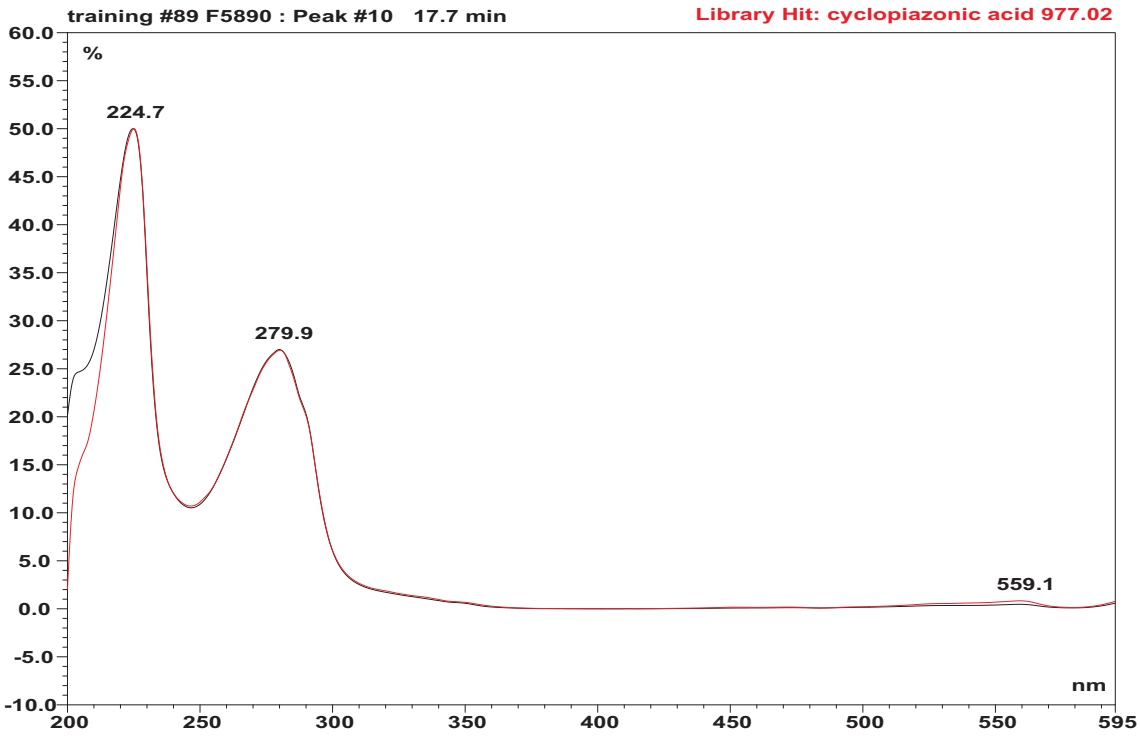


Figure 7.9: UV profile of compound NAM 7-9 (black) and the UV library hit (red) with the known α-cyclopiazonic acid (**7.5**) in the HPLC-UV/R_t library database.

The UV spectra of the other three compounds, NAM 7-6, 7-7 and 7-8 are shown in Figure 7.10 and none of them matched with known compounds when analyzed by the HPLC-UV/R_t library database.

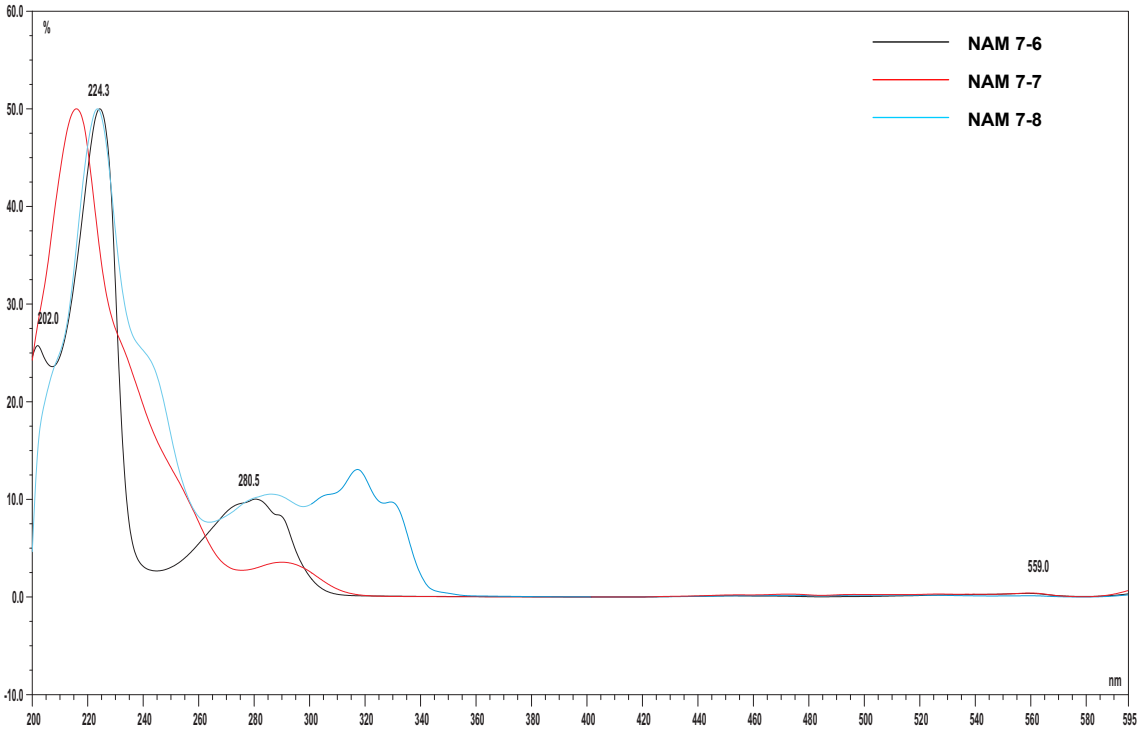
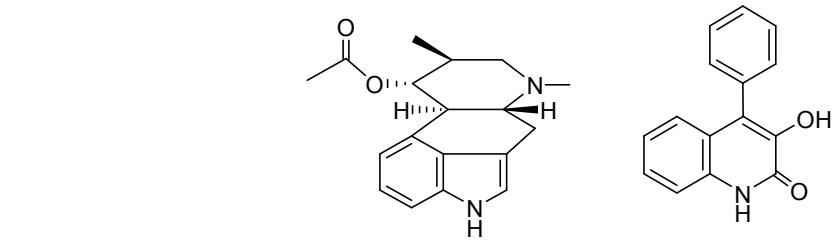
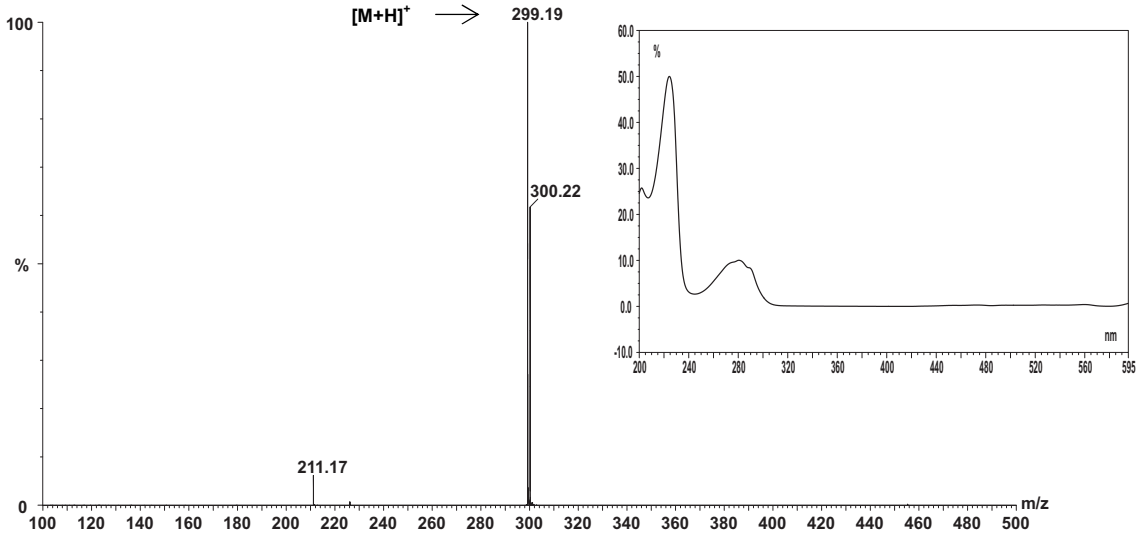


Figure 7.10: UV profile overlay for NAM 7-6, NAM 7-7 and NAM 7-8 of F5890.

The presence of a prominent $[M+H]^+$ signal and characteristic UV spectrum readily dereplicated compounds NAM 7-6 and NAM 7-8 as roquefortine A (**7.6**) and viridicatin (**7.8**), respectively based on their MS and UV spectral data search in the AntiMarin database and comparison with the published data by Nielsen and Smedsgaard (2003) (see Figure 7.11).



(7.6) **(7.8)**



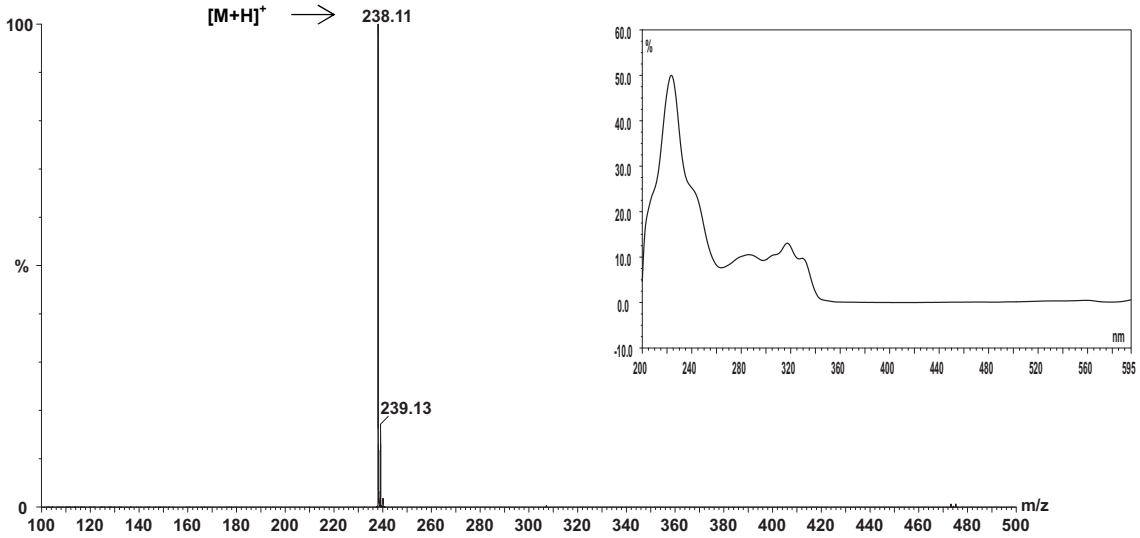


Figure 7.11: MS and UV spectral data of compounds NAM 7-6 (top) and NAM 7-8 (bottom). The MS data were obtained from TIC traces of TOFMS spectrum (positive ionization) of extract F5890.

Compound NAM 7-7 showed the presence of a dimeric cluster ion $[2M+H]^+$ that allowed assignment of the $[M+H]^+$ ion as 281 (see Figure 7.12). A search in the AntiMarin database on compounds with molecular weight of 279 – 281 Da resulted in 150 hits. Compound NAM 7-7 matched cyclopeptin (**7.7**) based on both their MS and UV spectra (Nielsen and Smedsgaard, 2003). It is interesting to mention here that the AntiMarin database could not readily dereplicate peak NAM 7-7 as cyclopeptin (**7.7**). Although the known compound (**7.7**) was one of the 150 hits in the AntiMarin database, the UV spectral data was not available (see Figure 7.13). Further searches using both the UV and MS spectral data of compound NAM 7-7 in the published database for fungal metabolites (Nielsen and Smedsgaard, 2003) resulted in one match with cyclopeptin (**7.7**) (see Figure 7.14).

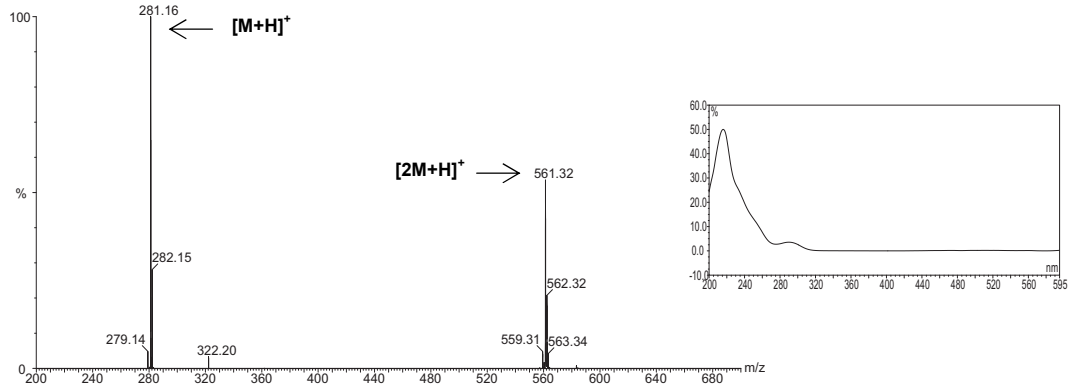
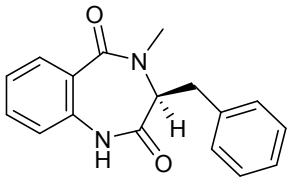


Figure 7.12: MS and UV spectral data of compound NAM 7-7. The MS was obtained from TIC traces of TOFMS spectrum (positive ionization) of extract F5890.



(7.7)

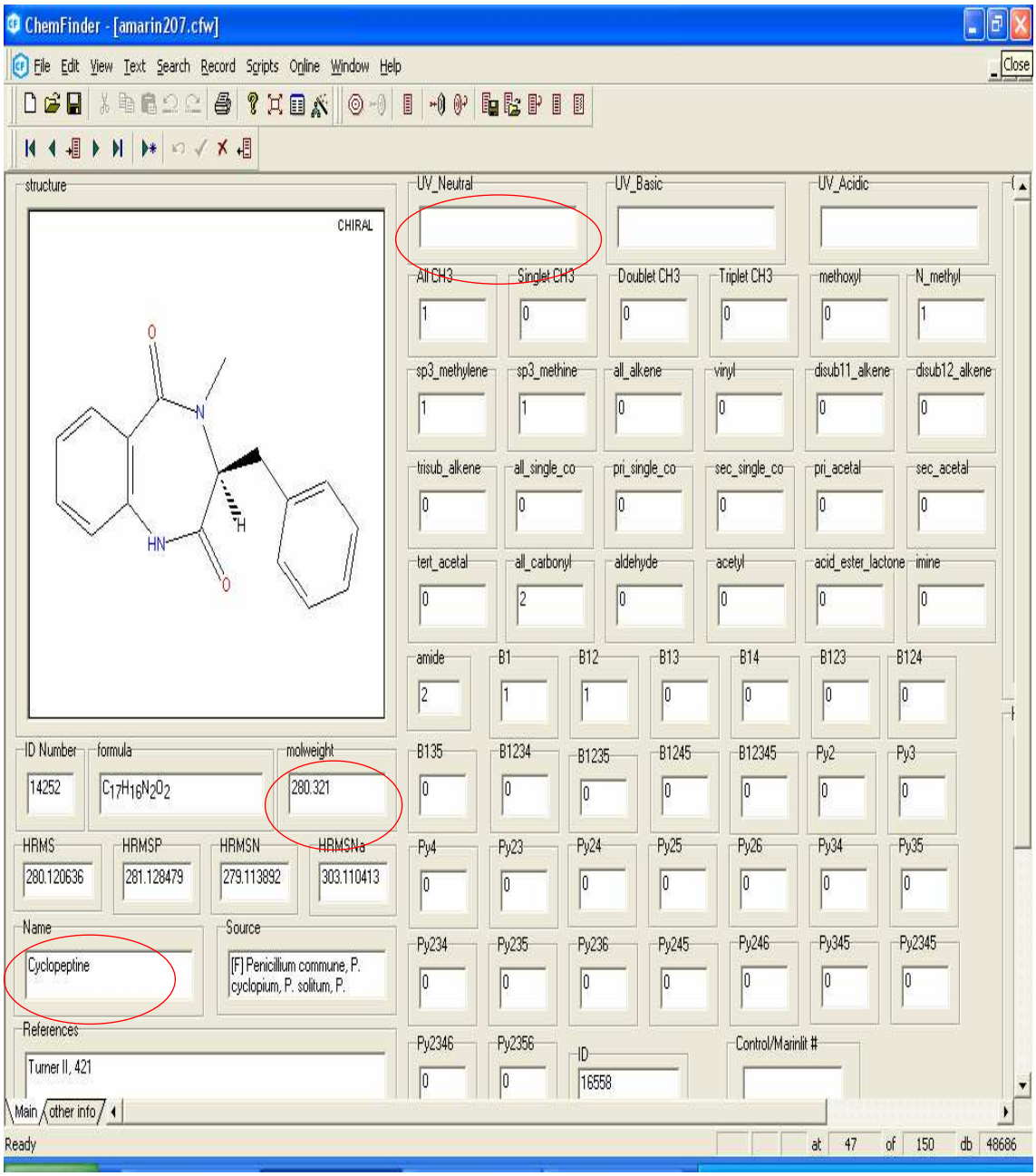
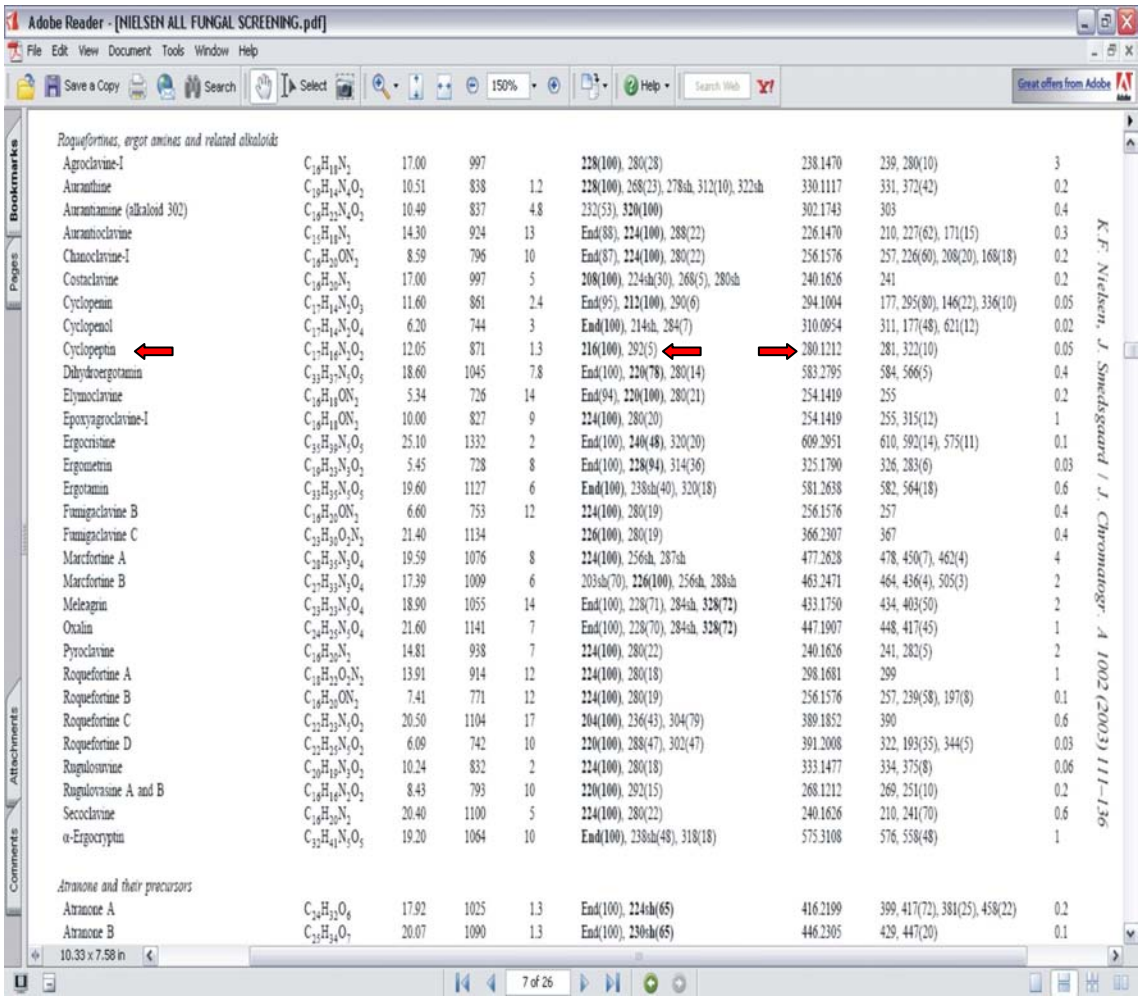


Figure 7.13: A screenshot of the AntiMarin database search for compound NAM 7-7 showing one of the 150 hits with known compound, cyclopeptin (**7.7**). The AntiMarin database did not contain the UV spectral data for cyclopeptin.



Roquefortines, ergot amines and related alkaloids								
Agroclavine-I	$C_{18}H_{18}N_2$	17.00	997		**228(100)**, 280(28)	238.1470	239, 280(10)	3
Aurantiine	$C_{19}H_{14}N_4O_2$	10.51	838	1.2	**228(100)**, 268(23), 278sh, 312(10), 322sh	330.1117	331, 372(42)	0.2
Aurantiamine (alkaloid 302)	$C_{18}H_{22}N_4O_2$	10.49	837	4.8	232(53), **320(100)**	302.1743	303	0.4
Aurantioclavine	$C_{17}H_{18}N_2$	14.30	924	13	End(88), **224(100)**, 288(22)	226.1470	210, 227(62), 171(15)	0.3
Chanoclavine-I	$C_{18}H_{20}ON_2$	8.59	796	10	End(87), **224(100)**, 280(22)	256.1576	257, 226(60), 208(20), 168(18)	0.2
Costaclavine	$C_{18}H_{20}N_2$	17.00	997	5	**208(100)**, 224sh(30), 268(5), 280sh	240.1626	241	0.2
Cyclopenin	$C_{17}H_{14}N_2O_3$	11.60	861	2.4	End(95), **212(100)**, 290(6)	294.1004	177, 295(80), 146(22), 336(10)	0.05
Cyclopenol	$C_{17}H_{14}N_2O_4$	6.20	744	3	**End(100)**, 214sh, 284(7)	310.0954	311, 177(48), 621(12)	0.02
Cyclopeptin	$C_{17}H_{16}N_2O_2$	12.05	871	1.3	**216(100)**, 292(5)	280.1212	281, 322(10)	0.05
Dihydroergotamin	$C_{33}H_{37}N_5O_5$	18.60	1045	7.8	End(100), **220(78)**, 280(14)	583.2795	584, 566(5)	0.4
Elymoclavine	$C_{16}H_{18}ON_2$	5.34	726	14	End(94), **220(100)**, 280(21)	254.1419	255	0.2
Epoxyagroclavine-I	$C_{16}H_{18}ON_2$	10.00	827	9	**224(100)**, 280(20)	254.1419	255, 315(12)	1
Ergocristine	$C_{35}H_{39}N_5O_5$	25.10	1332	2	End(100), **240(48)**, 320(20)	609.2951	610, 592(14), 575(11)	0.1
Ergometrin	$C_{19}H_{23}N_3O_2$	5.45	728	8	End(100), **228(94)**, 314(36)	325.1790	326, 283(6)	0.03
Ergotamin	$C_{33}H_{35}N_5O_5$	19.60	1127	6	**End(100)**, 238sh(40), 320(18)	581.2638	582, 564(18)	0.6
Fumigaclavine B	$C_{16}H_{20}ON_2$	6.60	753	12	**224(100)**, 280(19)	256.1576	257	0.4
Fumigaclavine C	$C_{23}H_{30}O_2N_2$	21.40	1134		**226(100)**, 280(19)	366.2307	367	0.4
Marcfortine A	$C_{28}H_{35}N_3O_4$	19.59	1076	8	**224(100)**, 256sh, 287sh	477.2628	478, 450(7), 462(4)	4
Marcfortine B	$C_{27}H_{33}N_3O_4$	17.39	1009	6	203sh(70), **226(100)**, 256sh, 288sh	463.2471	464, 436(4), 505(3)	2
Meleagrin	$C_{23}H_{23}N_3O_4$	18.90	1055	14	End(100), 228(71), 284sh, **328(72)**	433.1750	434, 403(50)	2
Oxalin	$C_{24}H_{25}N_3O_4$	21.60	1141	7	End(100), 228(70), 284sh, **328(72)**	447.1907	448, 417(45)	1
Pyroclavine	$C_{16}H_{20}N_2$	14.81	938	7	**224(100)**, 280(22)	240.1626	241, 282(5)	2
Roquefortine A	$C_{18}H_{22}O_2N_2$	13.91	914	12	**224(100)**, 280(18)	298.1681	299	1
Roquefortine B	$C_{16}H_{20}ON_2$	7.41	771	12	**224(100)**, 280(19)	256.1576	257, 239(58), 197(8)	0.1
Roquefortine C	$C_{22}H_{23}N_3O_2$	20.50	1104	17	**204(100)**, 236(43), 304(79)	389.1852	390	0.6
Roquefortine D	$C_{22}H_{25}N_3O_2$	6.09	742	10	**220(100)**, 288(47), 302(47)	391.2008	322, 193(35), 344(5)	0.03
Rugulosuvine	$C_{20}H_{19}N_3O_2$	10.24	832	2	**224(100)**, 280(18)	333.1477	334, 375(8)	0.06
Rugulovasine A and B	$C_{16}H_{16}N_2O_2$	8.43	793	10	**220(100)**, 292(15)	268.1212	269, 251(10)	0.2
Secoclavine	$C_{16}H_{20}N_2$	20.40	1100	5	**224(100)**, 280(22)	240.1626	210, 241(70)	0.6
α-Ergocryptin	$C_{32}H_{41}N_5O_5$	19.20	1064	10	**End(100)**, 238sh(48), 318(18)	575.3108	576, 558(48)	1
Atranone and their precursors								
Atranone A	$C_{24}H_{32}O_6$	17.92	1025	1.3	End(100), **224sh(65)**	416.2199	399, 417(72), 381(25), 458(22)	0.2
Atranone B	$C_{22}H_{34}O_7$	20.07	1090	1.3	End(100), **230sh(65)**	446.2305	429, 447(20)	0.1

Figure 7.14: A screenshot of a published data for fungal metabolites (Nielsen and Smedsgaard, 2003) showing the search result for compound NAM 7-7 with cyclopeptin (**7.7**).

7.2.4 Extract F5350

Extract F5350 was obtained from the small scale extraction of isolate KK3T8 grown on PYGA medium. This small-scale extract showed fair cytotoxicity (IC_{50}5,403 ng/mL) and was active against *C. albicans* (>70% inhibition). The HPLC screening of F5350 revealed one main compound, NAM 7-10 eluted over the bioactive region (see Figure 7.15).

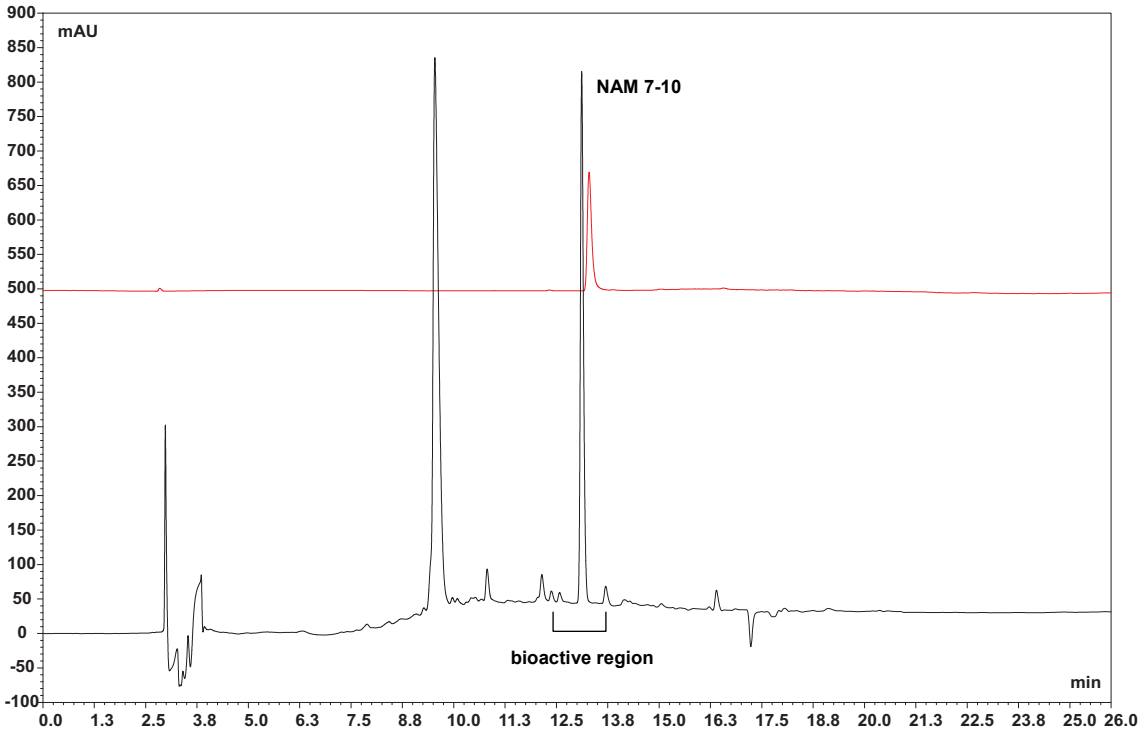


Figure 7.15: HPLC chromatogram of F5350 showing overlay of ELSD detection (top) for compound NAM 7-10 over the bioactive region.

Compound NAM 7-10 showed the presence of a dimeric cluster ion $[2M+H]^+$ that allowed assignment of the $[M+H]^+$ ion as 281 (see Figure 7.16). A search in the AntiMarin database on compounds with molecular weight of 279 – 281 Da resulted in 150 hits. Compound NAM 7-10 matched brefeldin A (**7.10**) based on both the MS and UV spectral data (Nielsen and Smedsgaard, 2003).

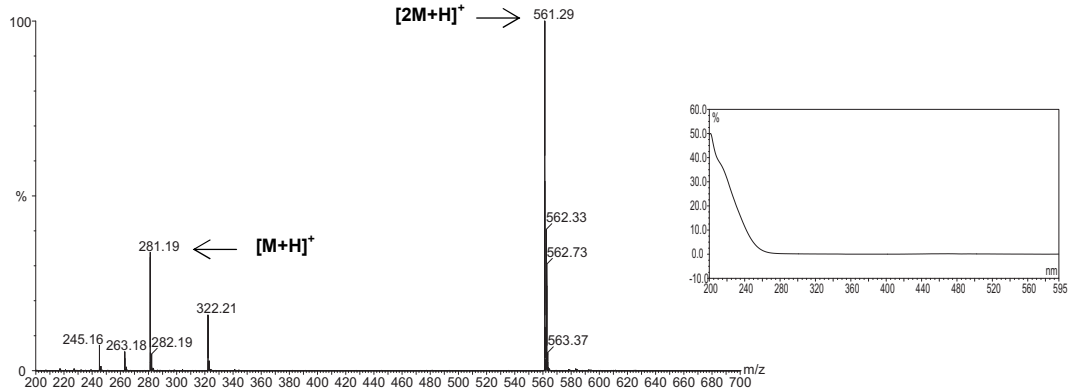
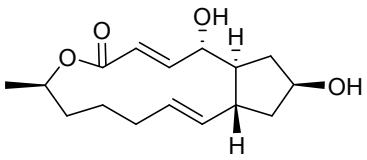


Figure 7.16: MS and UV spectral data of compound NAM 7-10. The MS was obtained from TIC traces of TOFMS spectrum (positive ionization) of extract F5350.



(7.10)

7.2.5 Extract F5868

Extract F5868 was obtained from the small scale extraction of isolate KK4T14b grown on PYGA medium. This small-scale extract showed good cytotoxicity (IC_{50}383 ng/mL) but was inactive in the antimicrobial assays. The HPLC screening of F5868 revealed two main compounds, NAM 7-11 and NAM 7-12 eluted over the cytotoxic region (11.0 – 15.0 min) (see Figure 7.17).

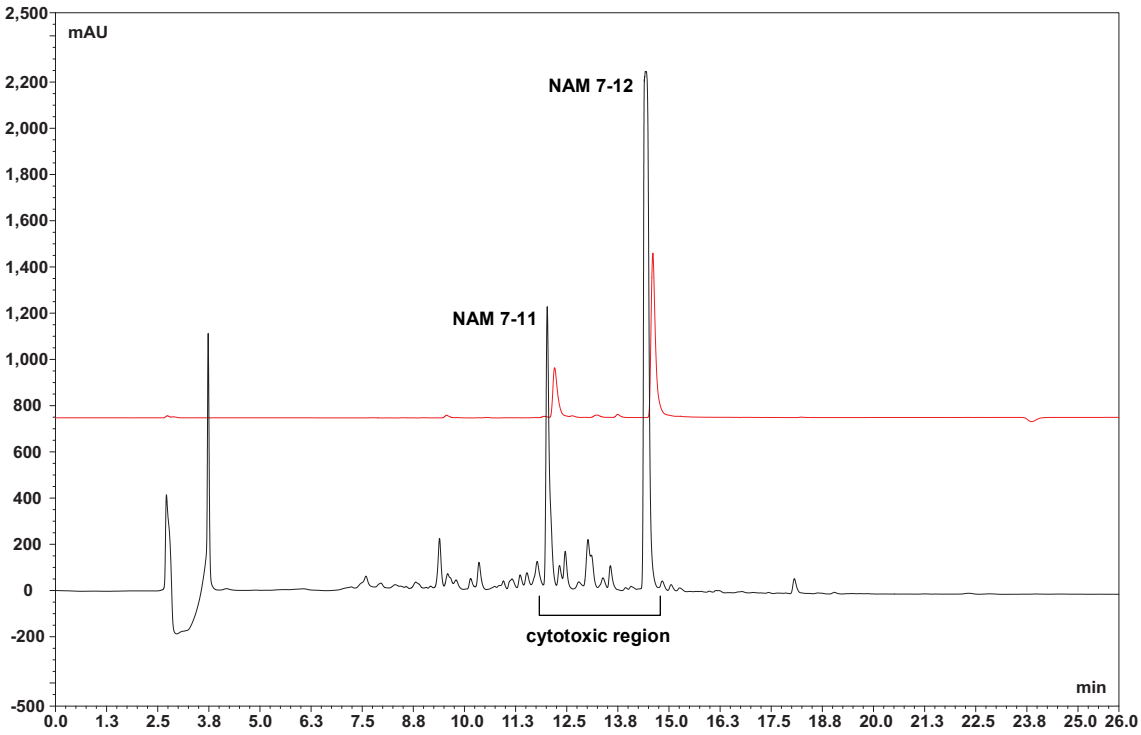


Figure 7.17: HPLC chromatogram of F5868 showing overlay of ELSD detection (top) for compounds NAM 7-11 and NAM 7-12 over the cytotoxic region.

The UV spectra of compounds NAM 7-11 and NAM 7-12 are shown in Figure 7.18. Compound NAM 7-12 showed an exact match with the known compound, mycophenolic acid (**7.12**), while no hits were found for compound NAM 7-11 from the HPLC-UV/R_t library database.

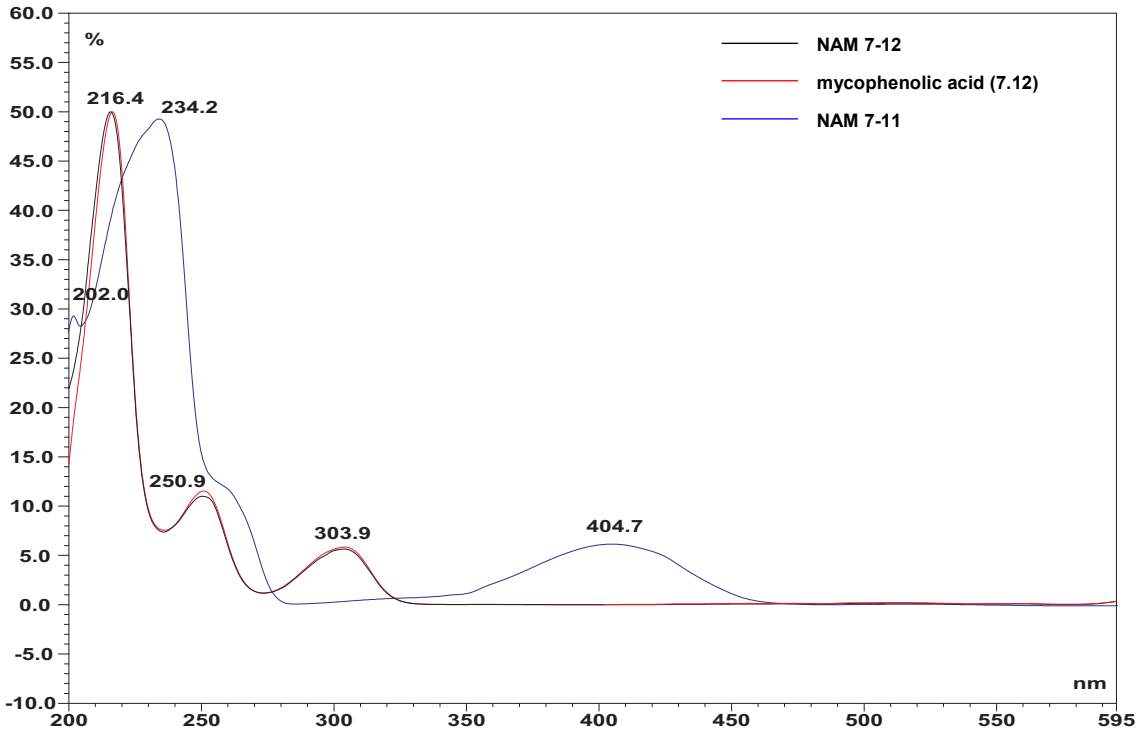
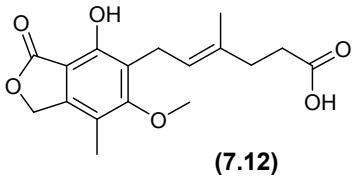


Figure 7.18: UV profile of F5868 showing overlay of compound NAM 7-12 (black) and the UV library hit (red) with the known mycophenolic acid (**7.12**) in the HPLC-UV/R_t library database. An overlay of the UV spectrum of compound NAM 7-11 is shown in blue.



(7.12)

The MS data of compound NAM 7-11 (Figure 7.19) indicated the mass to be 366 Da ($[M+H]^+$). A search in the AntiMarin database for compounds with molecular weight of 364 – 366 Da resulted in 223 hits. Further search in the published database on fungal metabolites (Nielsen and Smeedsgaard, 2003) resulted in a match in both UV and MS data with the known brevianamide A (**7.11**).

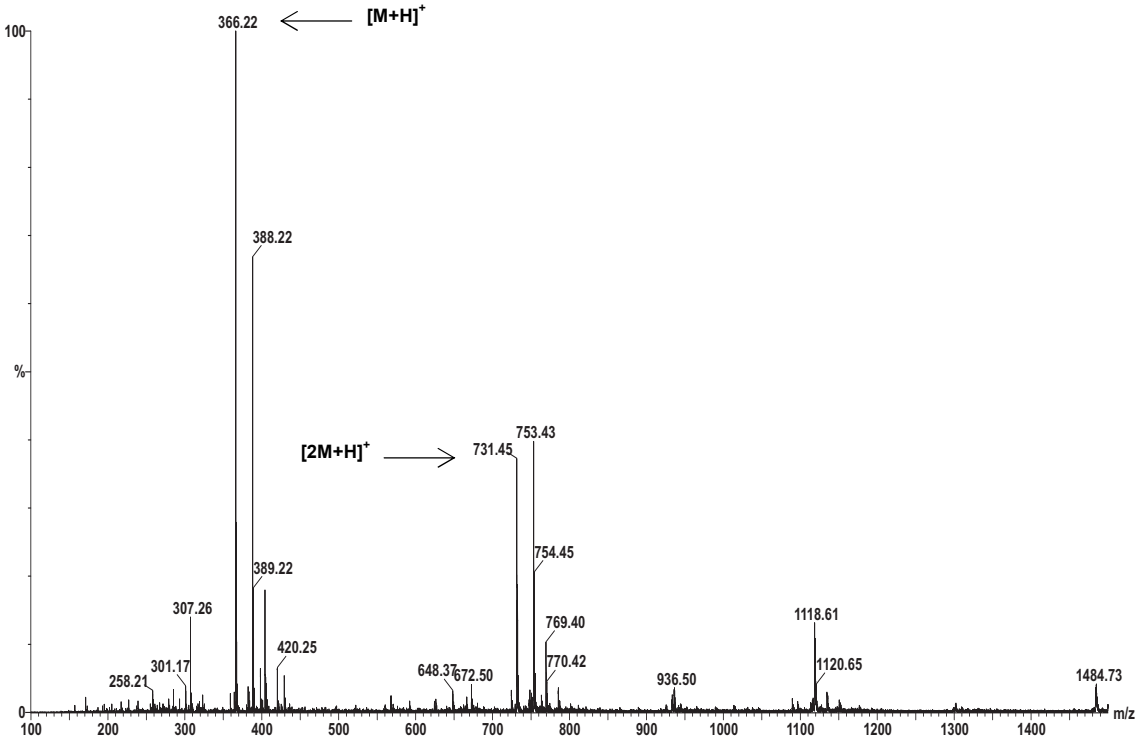
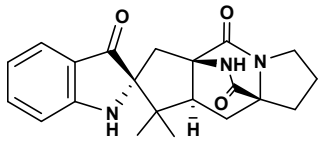


Figure 7.19: ESIMS spectrum of compound NAM 7-11 obtained from F5868.



(7.11)

7.3 Dereplication using CapNMR technique

Significant peaks in two extracts that were not readily dereplicated using the HPLC-UV/R_t library database and HPLC-MS-UV were investigated using the CapNMR technique. These were extracts F5962 and F6430 discussed in **Sections 7.3.1** and **7.3.2**, respectively. Details of the procedures were discussed in **Chapter Two** (*Experimental*).

7.3.1 Extract F5962

Extract F5962 was obtained from a SCA culture medium of *Streptomyces* sp. (LA5L4). This small-scale extract showed activity against P388 cells (IC_{50}1,339 ng/mL) but was inactive in the antimicrobial assays. The HPLC screening of F5962 revealed two main peaks, NAM 7-13 and NAM 7-14, which were eluted over the cytotoxic region (see Figure 7.20).

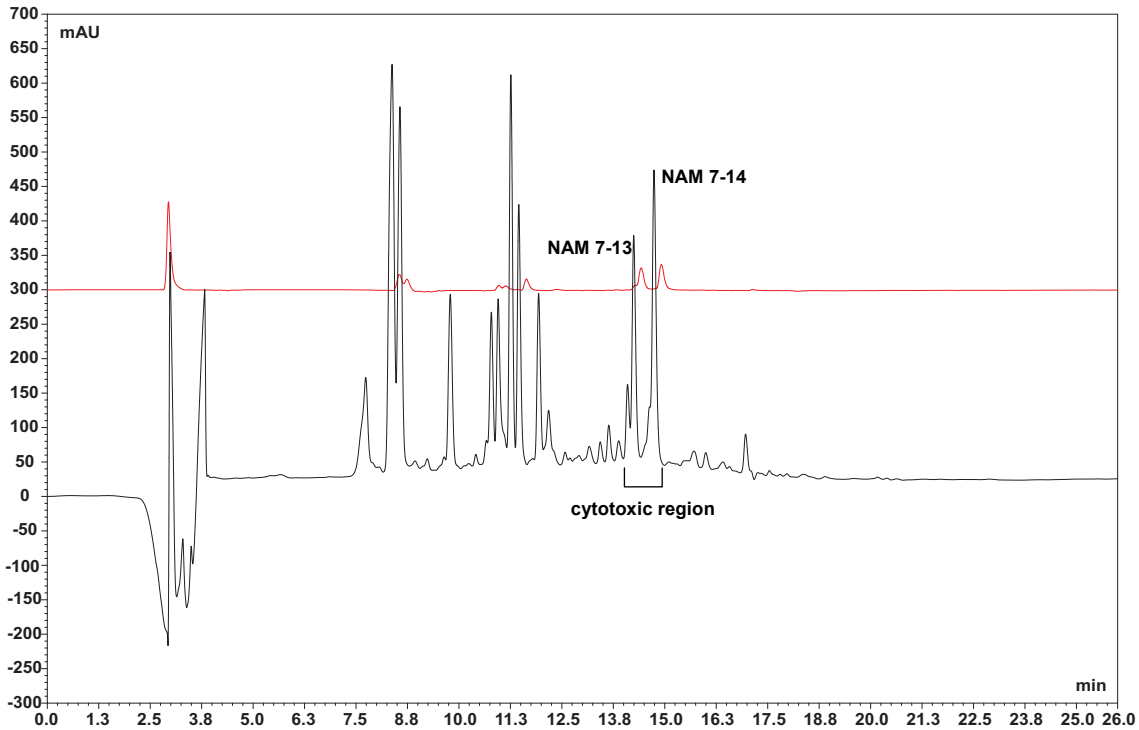


Figure 7.20: HPLC chromatogram of F5962 showing overlay of ELSD detection (top) for compounds NAM 7-13 and NAM 7-14 over the cytotoxic region.

These two peaks were shown to have virtually identical UV chromophores, suggesting that they were closely related in structure (Figure 7.21). The TIC traces from the HPLC TOFMS spectrum of F5962 showed that compounds NAM 7-13 and NAM 7-14 had an identical molecular mass of 339 Da ($[M+H]^+$). A search in the AntiMarin database for compounds with molecular mass of 337 – 339 Da, revealed 218 matches and the structures could not be readily dereplicated. An attempt to purify these two compounds for the CapNMR analysis resulted in partially pure compound NAM 7-14, however no suitable amount of compound NAM 7-13 was obtained in the attempt.

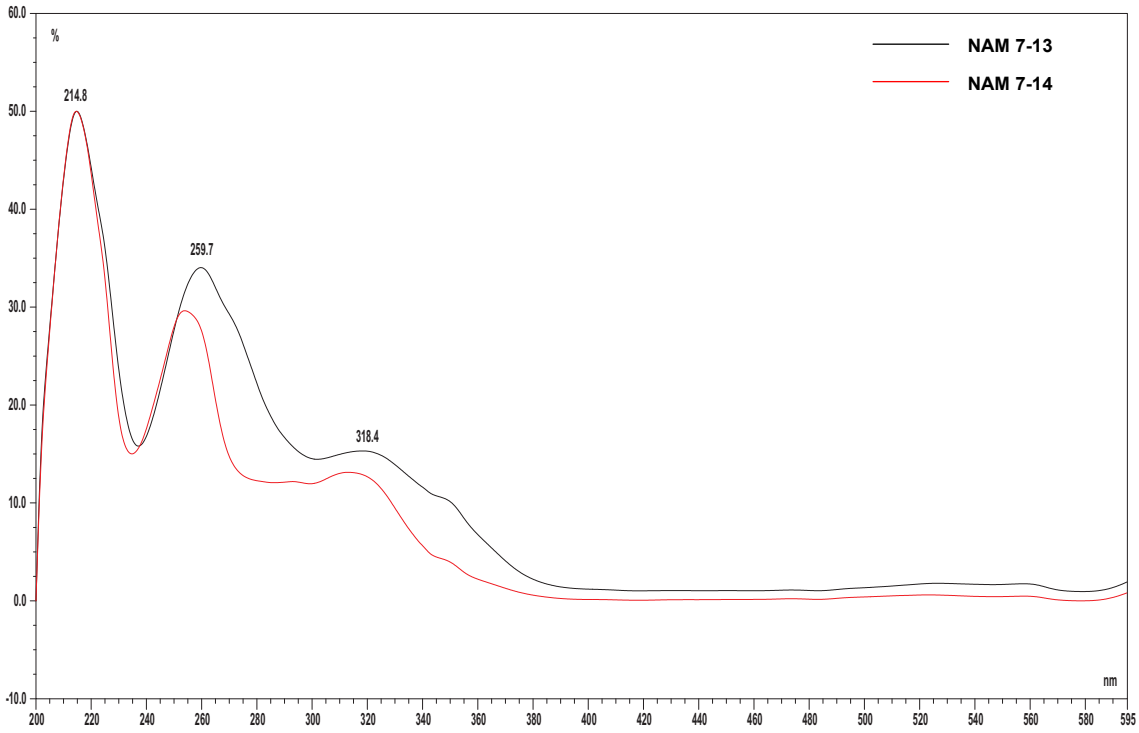


Figure 7.21: UV profile overlay for compounds NAM 7-13 and NAM 7-14 obtained from F5962.

Approximately 2 µg of compound NAM 7-14 was analyzed using the CapNMR. The ^{1}H NMR spectrum of compound NAM 7-14 (see Figure 7.22) indicated the presence four aromatic protons appearing at δ_H 6.8 – 7.6, a singlet methyl (δ_H 1.45) and another singlet methyl (δ_H 2.51) that could indicate an N-methyl or an aromatic methyl. The ESIMS data of compound NAM 7-14, shown in Figure 7.23 confirmed the mass to be 339 Da ($[M+H]^+$). Searching in the AntiMarin database on two singlet methyls and a molecular weight of 337 – 339 Da resulted in 19 hits (of 218 matches without the ^{1}H NMR data input). The indication of the presence of an N-methyl or aromatic methyl in compound NAM 7-14 (δ_H 2.51) was sufficient in reducing the 19 known compounds to two; namely thiazostatin A (**7.13**) and thiazostatin B (**7.14**).

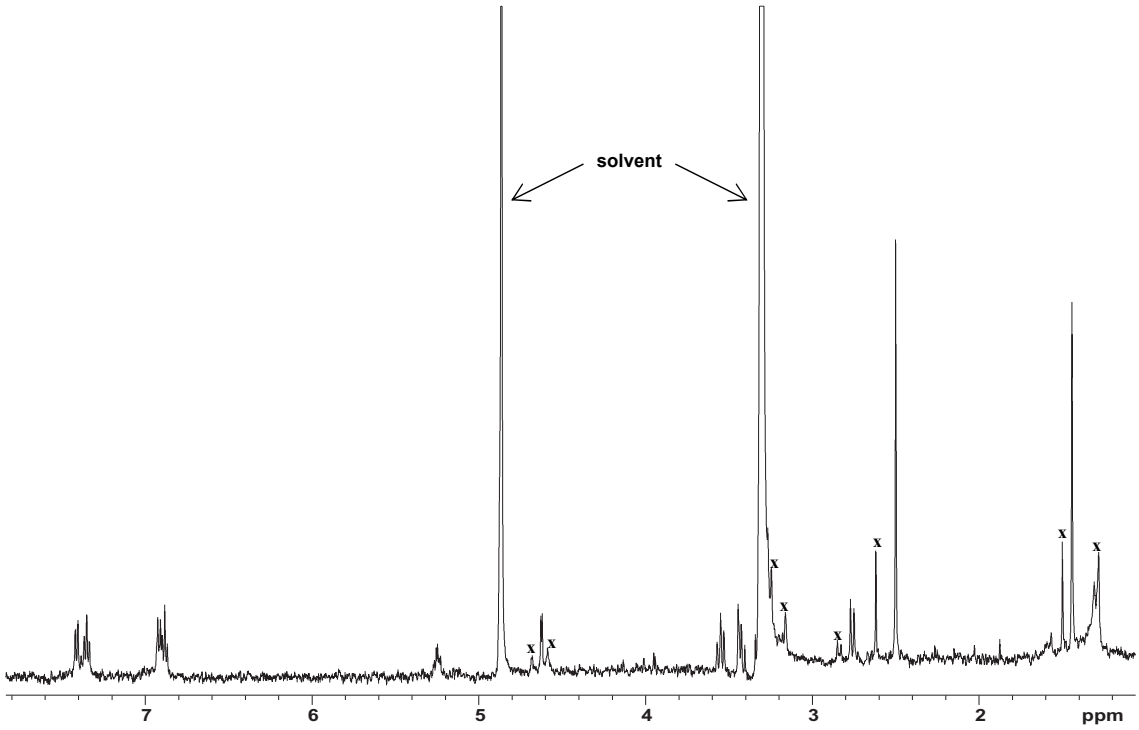


Figure 7.22: ^{1}H NMR spectrum of compound NAM 7-14 in CD_3OD obtained from F5962. Crosses indicate impurities.

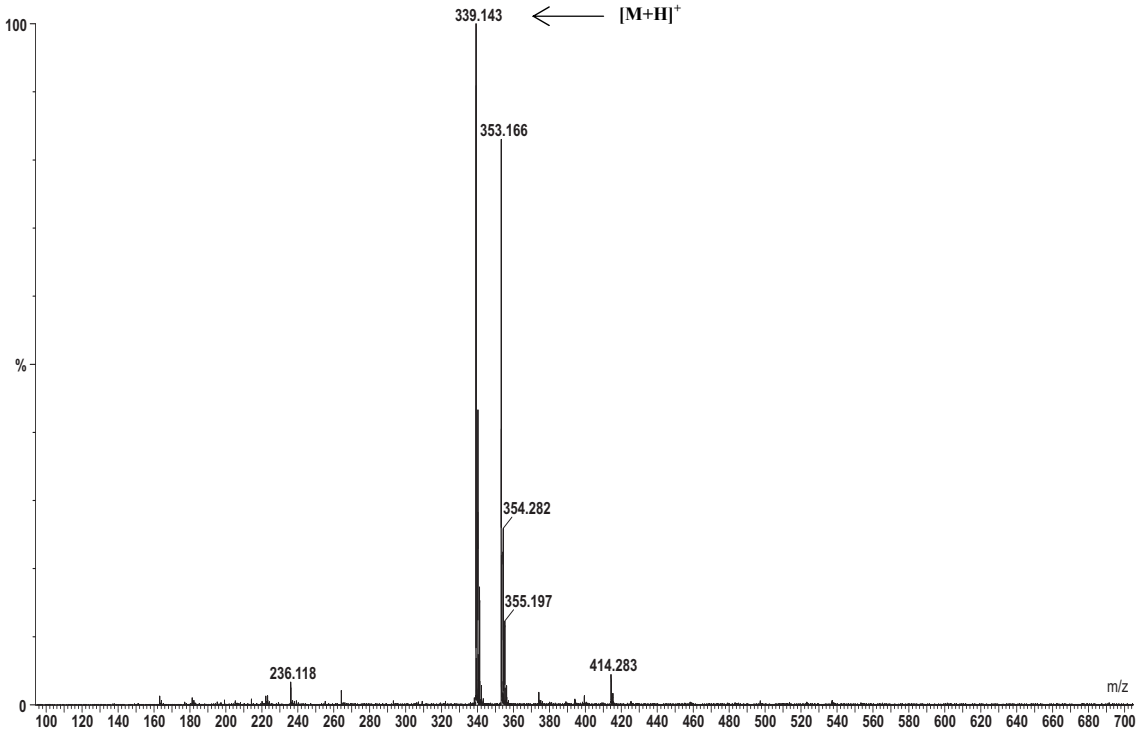
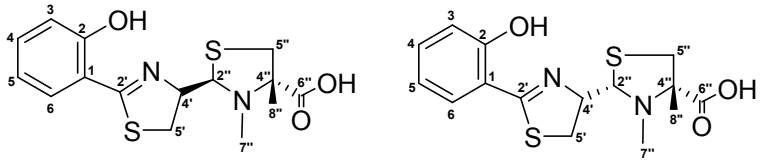


Figure 7.23: ESIMS spectrum of compound NAM 7-14.



(7.13) **(7.14)**

The ^{1}H NMR data of compound NAM 7-14 were compared to those reported for **7.13** and **7.14** (Shindo *et al*., 1989) and these are shown in Table 7.1. It was noted that the ^{1}H NMR spectrum of compound NAM 7-14 presented in Figure 7.22 also contained some signals of thiazostatin A (**7.13**). Based on the comparison in Table 7.1, it was deduced that the data of compound NAM 7-14 were more comparable to those for **7.14** rather than to those reported for **7.13**. Based on these data, compound NAM 7-14 was identified as thiazostatin B (**7.14**).

Table 7.1: Comparison of ^{1}H NMR data of compound NAM 7-14 and the reported compounds **7.13** and **7.14**.

Position	δ^{1}H, ppm multiplicity (J_{HH} Hz)		
	NAM 7-14[a]	**7.13**[b]	**7.14**[b]
1			
2			
3	6.92, d (8.3)[c]	6.92, dd (0.9, 8.5)	6.93, dd (0.9, 8.5)
4	7.35, t (8.3, 8.3)[c]	7.35, ddd (1.5, 7.8, 8.5)	7.35, ddd (1.6, 7.8, 8.5)
5	6.88, t (8.3, 8.3)[c]	6.88, ddd (0.9, 7.8, 7.8)	6.88, ddd (0.9, 7.8, 7.8)
6	7.41, d (8.3)[c]	7.39, dd (1.5, 7.8)	7.41, dd (1.6, 7.8)
2'			
4'	5.23 (m)	5.14, dt (3.2, 9.1)	5.23, dt (3.4, 9.0)
5'	3.41, dd (9.4, 11.2)	3.28, dd (9.1, 11.0)	3.41, dd (9.0, 11.2)
	3.53, dd (9.4, 11.2)[c]	3.54, dd (9.1, 11.0)	3.53, dd (9.1, 11.2)
2"	4.68, d (4.2)[c]	4.68, d (3.2)	4.68, d (3.4)
4"			
5"	2.79, d (10.6)	2.85, d (10.6)	2.79, d (12.0)
	3.34[d]	3.17, d (10.6)	3.34, d (12.0)
6"			
7"	2.51, s (CH_3)	2.63, s (CH_3)	2.50, s (CH_3)
8"	1.45, s (CH_3)	1.50, s (CH_3)	1.45, s (CH_3)

[a] These data were recorded at 500 MHz in CD_3OD; [b] These data were recorded at 400 MHz in CD_3OD (Shindo *et al*., 1989). [c] These values were observed as overlapping signals of compounds (**7.13**) and (**7.14**). [d] This value was not fully resolved.

7.3.2 Extract F6430

Extract F6430 was obtained from a PYGB culture medium of *Paecilomyces* sp. (PR5L9). This small-scale extract showed a moderately cytotoxic activity against P388 cells (IC_{50}10,445 ng/mL) but was inactive in the antimicrobial assays.. The HPLC screening of F6430 revealed five main compounds, NAM 7-15, NAM 7-16, NAM 7-17, NAM 7-18 and NAM 7-19 which eluted over 10.0 – 16.0 min (see Figure 7.24).

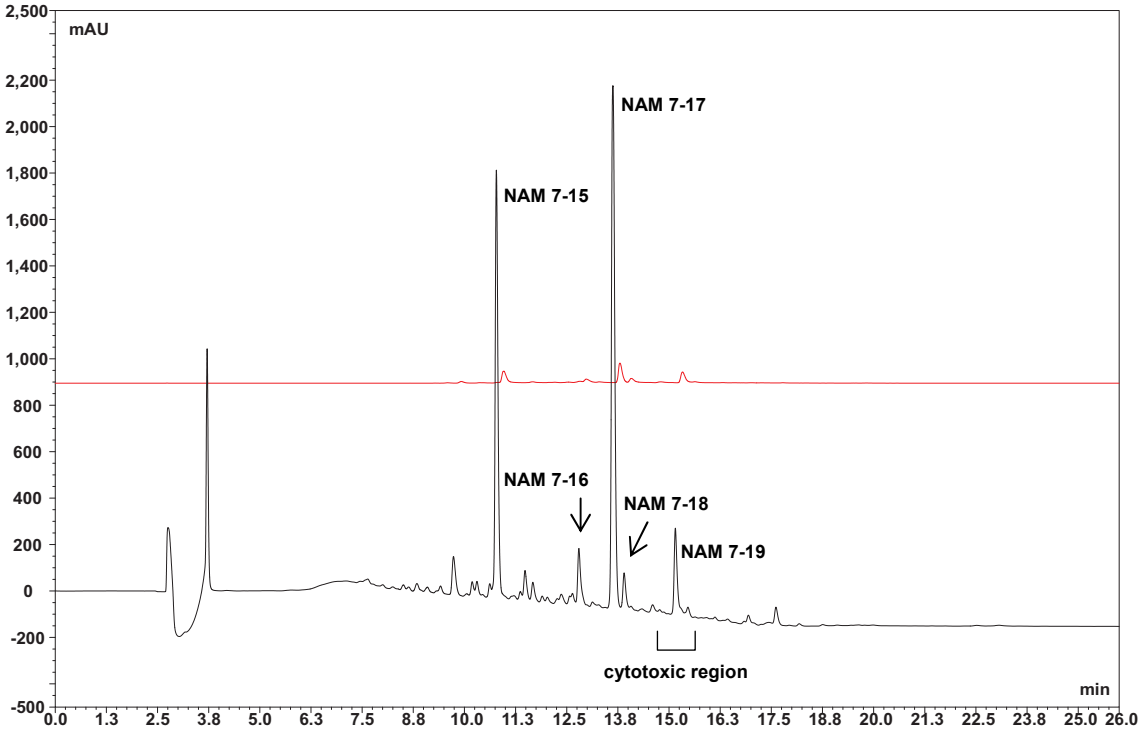


Figure 7.24: HPLC chromatogram of F6430 showing overlay of ELSD detection (top) for compounds NAM 7-15, NAM 7-16, NAM 7-17, NAM 7-18 and NAM 7-19.

The HPLC fractionation of 250 µg of crude extract (F6430) gave partially purified compounds NAM 7-15, NAM 7-16, NAM 7-17, NAM 7-18 and NAM 7-19 (see Figure 7.25). Characterization of these compounds is discussed in **Section 7.3.2.1** (NAM 7-15 and NAM 7-17) and **Section 7.3.2.2** (NAM 7-16). Compounds NAM 7-18 and NAM 7-19 are not further discussed as insufficient high quality NMR data were obtained.

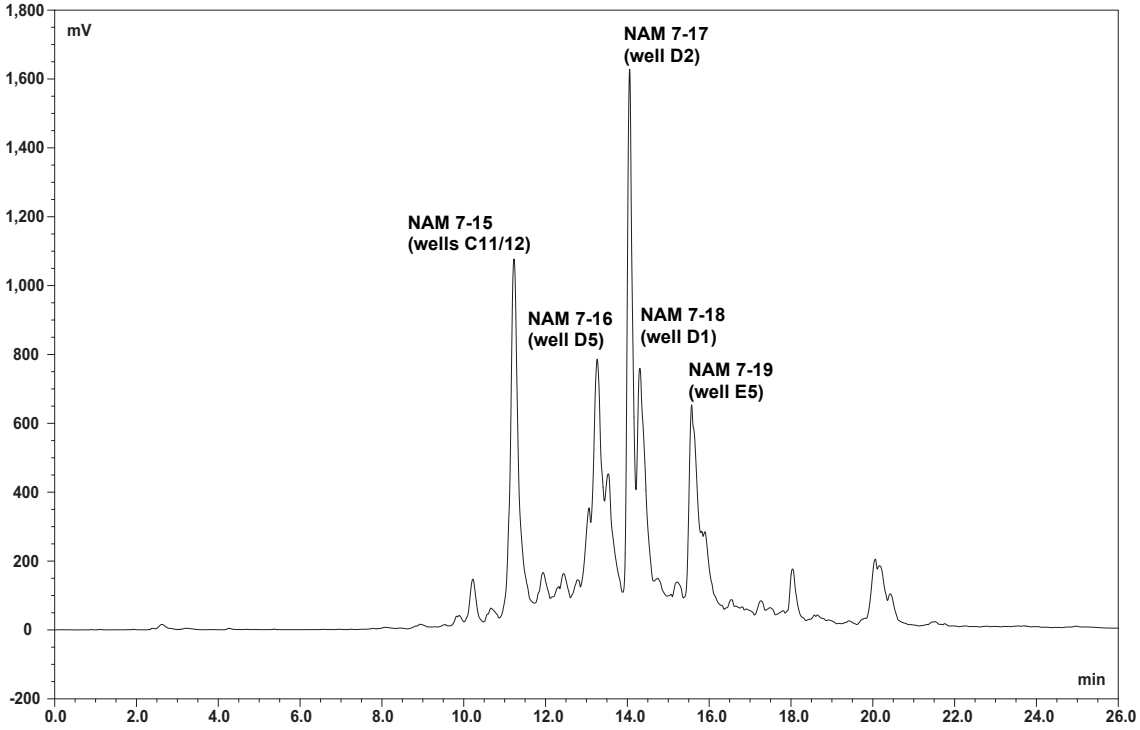


Figure 7.25: HPLC chromatogram of crude extract F6430 (250 µg) showing the fractionation of compounds NAM 7-15, NAM 7-16, NAM 7-17, NAM 7-18 and NAM 7-19 collected into their respective wells of the microtitre plate.

7.3.2.1 Compounds NAM 7-15 and NAM 7-17

Compounds NAM 7-15 and NAM 7-17 had identical UV chromophores (Figure 7.26), suggesting they were related structures. Compound NAM 7-15 showed an exact match in both UV profile and R_t with the known compound, indole-3-carboxylic acid (**7.15**) in the HPLC-UV/R_t library database. Compound NAM 7-17 also displayed a match with the UV spectrum of the indole-3-carboxylic acid (**7.15**) but the R_t differed significantly.

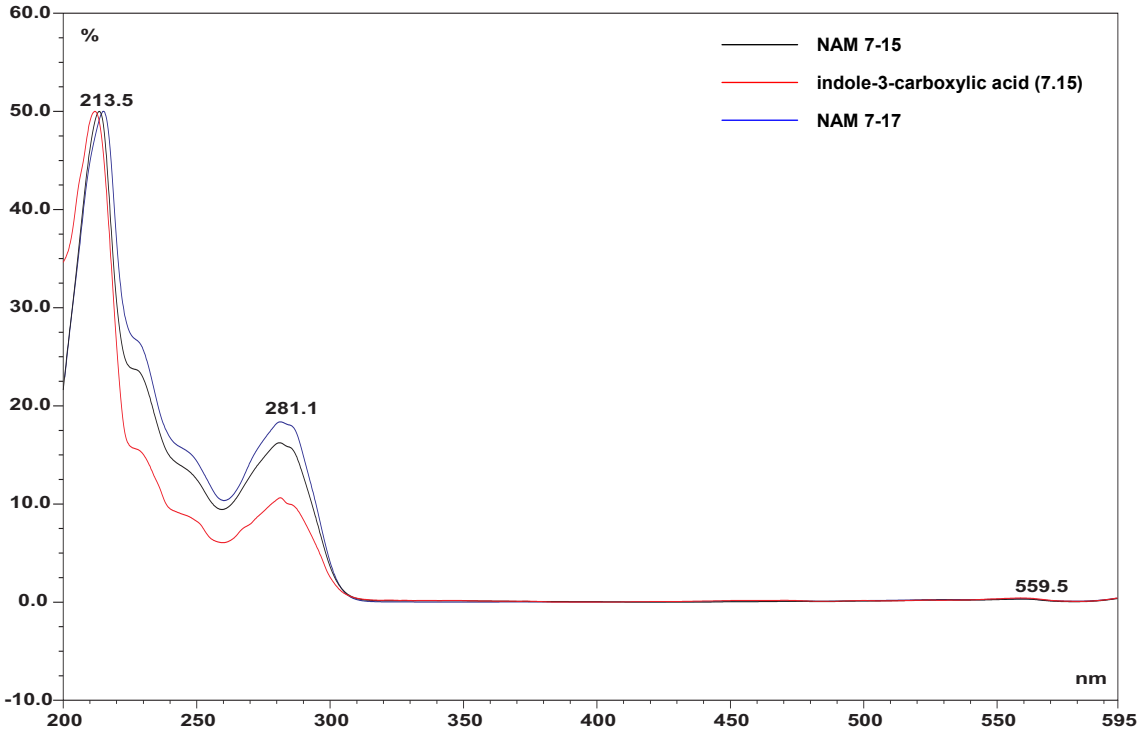
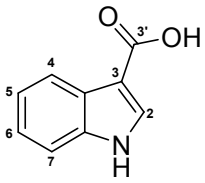


Figure 7.26: UV profile of F6430 showing overlays of compound NAM 7-15 (black) and the HPLC-UV/R_t library hit (red) with the known indole-3-carboxylic acid (**7.15**). An overlay of the UV spectrum of compound NAM 7-17 is shown in blue.



(7.15)

The ^{1}H NMR spectrum of compound NAM 7-17 (2 µg) revealed a singlet methyl group (δ_H 3.88), one singlet aromatic proton (δ_H 7.95) and another four vicinal coupled aromatic protons (δ_H 7.17 - 8.04) (see Figure 7.27).

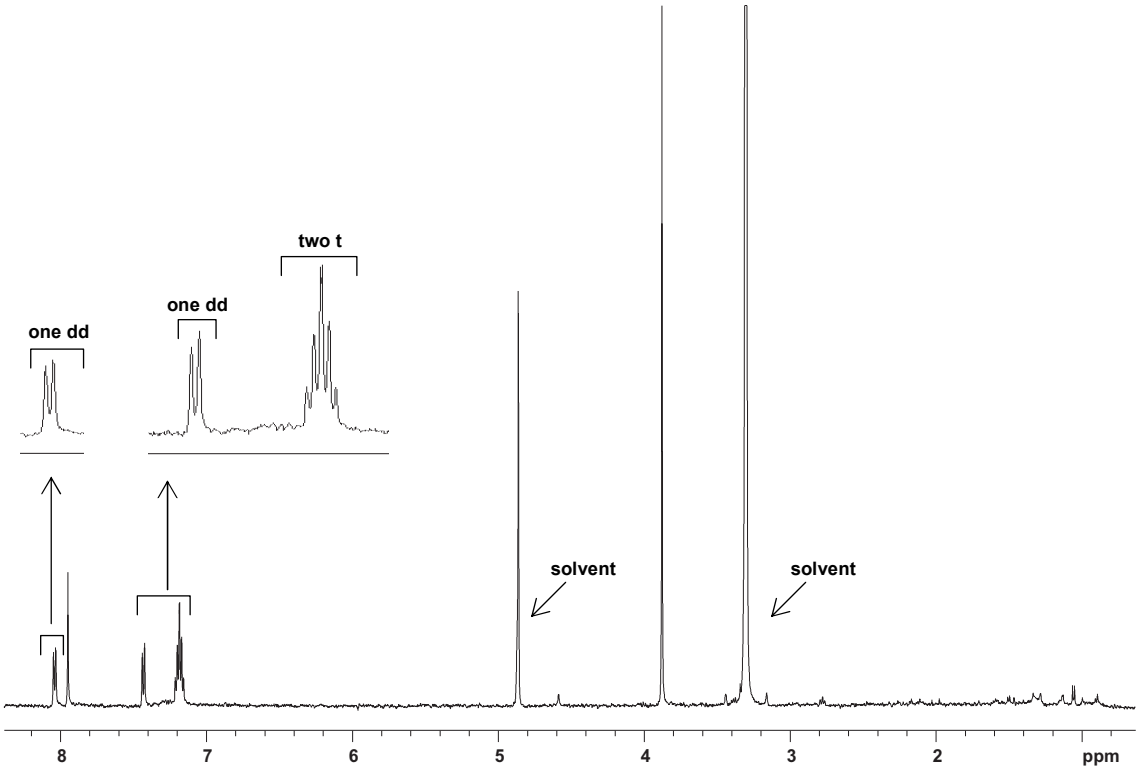


Figure 7.27: ^{1}H NMR spectrum of compound NAM 7-17 in CD_3OD obtained from F6430.

The ESIMS spectrum of compound NAM 7-17 (Figure 7.28) showed a molecular mass of 176 Da ($[M+H]^+$), indicating an additional 14 mass units compared to compound NAM 7-15 (**7.15**).

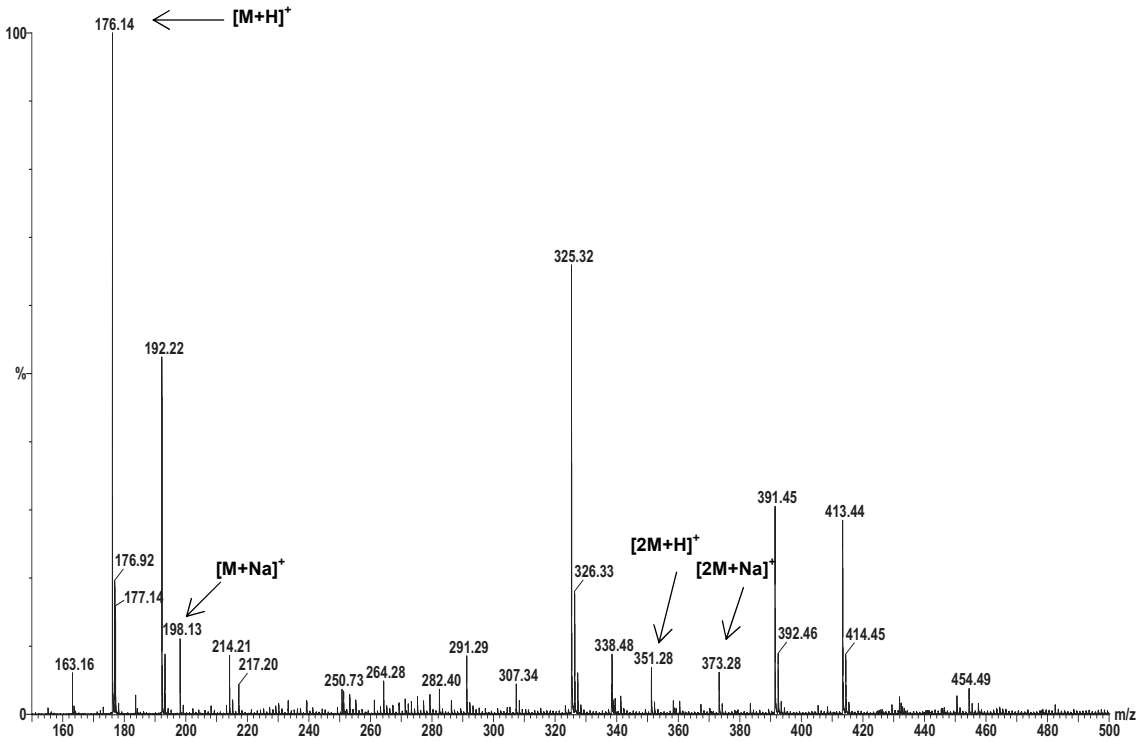
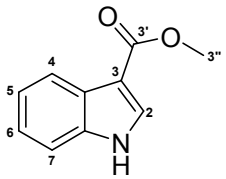
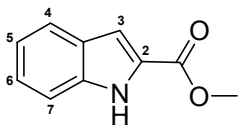


Figure 7.28: ESIMS spectrum of compound NAM 7-17.

A search in the AntiMarin database for indole-related compounds with molecular weight of 174 – 176 Da and a singlet methyl resulted in two hits; namely methyl 1*H*-indole-3-carboxylate (**7.17**) and methyl 1*H*-indole-2-carboxylate (**7.18**).



(7.17)



(7.18)

The ^{1}H NMR data of compound NAM 7-17 was compared with the reported compounds methyl 1*H*-indole-3-carboxylate (**7.17**) (Tan *et al*., 2004) and 1*H*-indole-3-carboxylic acid (**7.15**) (Yue *et al*., 2000). These are shown in Table 7.2. Variations in the values were expected between compounds NAM 7-17 and **7.17** since the chemical shifts of both compounds were referenced against different solvent peaks. The ^{1}H spectral data of compound NAM 7-17 (excluding the methoxyl protons) were consistent with a 3-substituted indole as described for the reported 1*H*-indole-3-carboxylic acid (**7.15**). The additional mass of 14 of compound NAM 7-17 compared to that of 1*H*-indole-3-carboxylic acid (**7.15**) corresponded to an additional methoxyl group. Based on these data, compound NAM 7-17 was identified as methyl 1*H*-indole-3-carboxylate (**7.17**).

Table 7.2: Comparison of ^{1}H NMR data of compound NAM 7-17 and of the reported compounds 7**.15** and **7.17**.

Position	**δ^{1}H, ppm multiplicity (J_{HH} Hz)**		
	NAM 7-17[a]	**7.15 [b]**	**7.17[c]**
1			
2	7.95, s	8.32, s	7.94, s
3			
4	8.04, d, br (7.3)	8.27, d (9.0)	8.05, d (7.7)
5	7.17, ddd (7.3, 7.3, 1.0)	7.25, m	7.16, ddd (7.7, 7.7, 1.2)
6	7.19, ddd (7.3, 7.3, 1.0)	7.25, m	7.19, ddd (7.7, 7.7, 1.2)
7	7.43 d, br (7.3)	7.54, d (9.0)	7.43, d (7.7)
3'	3.88, s	4.70, s	

[a] These data were recorded at 500 MHz in CD_3OD; [b] These data were recorded at 400 MHz in acetone-d_6 (Yue *et al*., 2000); [c] These data were recorded at 500 MHz in CD_3OD, reference signal value not stated (Tan *et al*., 2004).

7.3.2.2 Compound NAM 7-16

The UV spectrum of compound NAM 7-16 (see Figure 7.29) did not show any match with known compounds in the HPLC-UV/R_t library database. Although the ^{1}H NMR spectrum of compound NAM 7-16 (3 µg) indicated some impurities, the presence of a doublet methyl at δ_H 1.51 and two aromatic protons (δ_H 6.93 and 8.15) with an *ortho*-coupling pattern was clearly shown (see Figure 7.30).

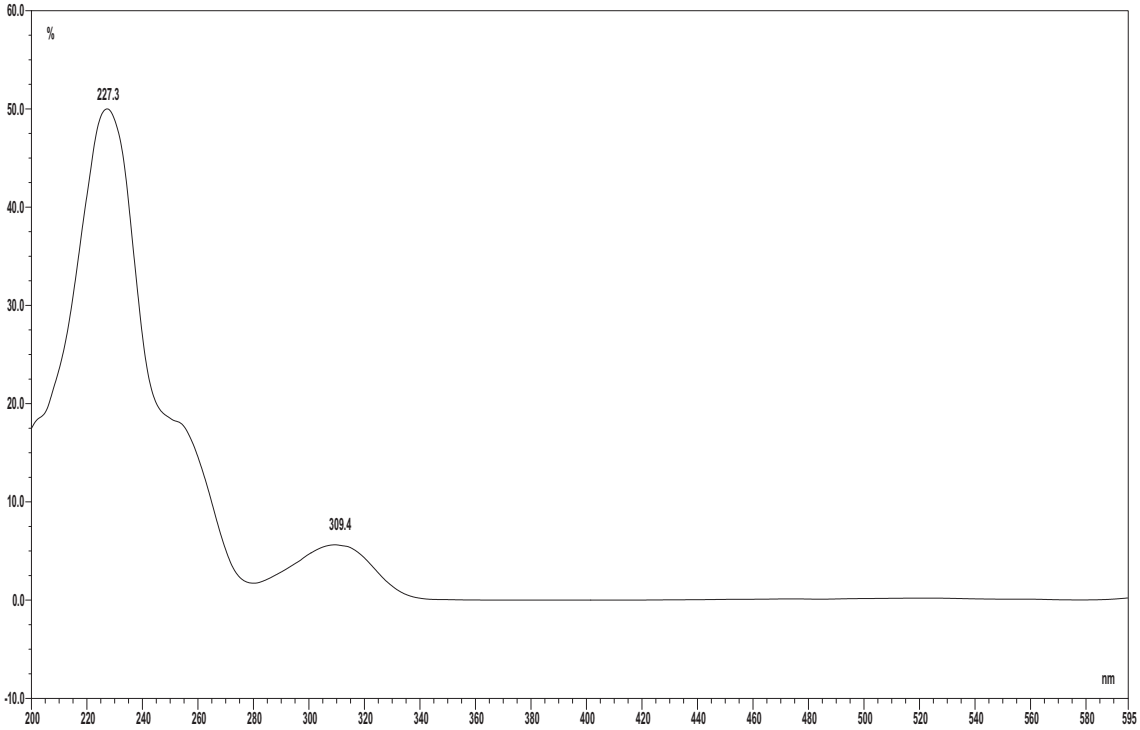


Figure 7.29: UV profile of NAM 7-16 obtained from F6430.

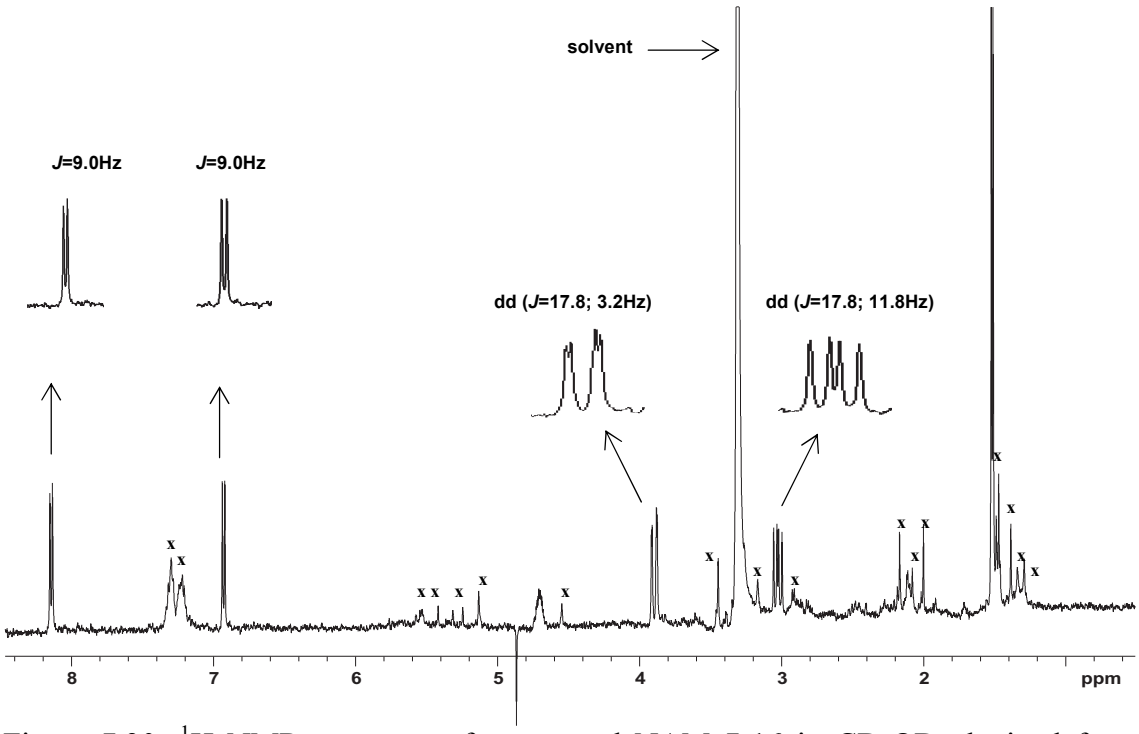


Figure 7.30: ^{1}H NMR spectrum of compound NAM 7-16 in CD_3OD obtained from F6430. Crosses indicate impurities.

Compound NAM 7-16 had a molecular mass of 223 Da ($[M+H]^+$) (Figure 7.31) and a search in the AntiMarin database on compounds with a molecular weight of 221 – 223 Da and a doublet methyl resulted in 14 hits (of 208 hits without the ^{1}H NMR data input). The coupling constant pattern of the aromatic protons in compound NAM 7-16 (δ_H 6.93 and 8.15) was helpful to discriminating against 12 of the 14 possible matches. The two compounds that possibly fit the structure for compound NAM 7-16 were (*R*)-8-hydroxyl-3-methyl-1-oxoisochroman-5-carboxylic acid (known as 5-carboxymellein) (**7.16**) and (*R*)-8-hydroxyl-3-methyl-1-oxoisochroman-7-carboxylic acid (known as ochratoxin β) (**7.19**).

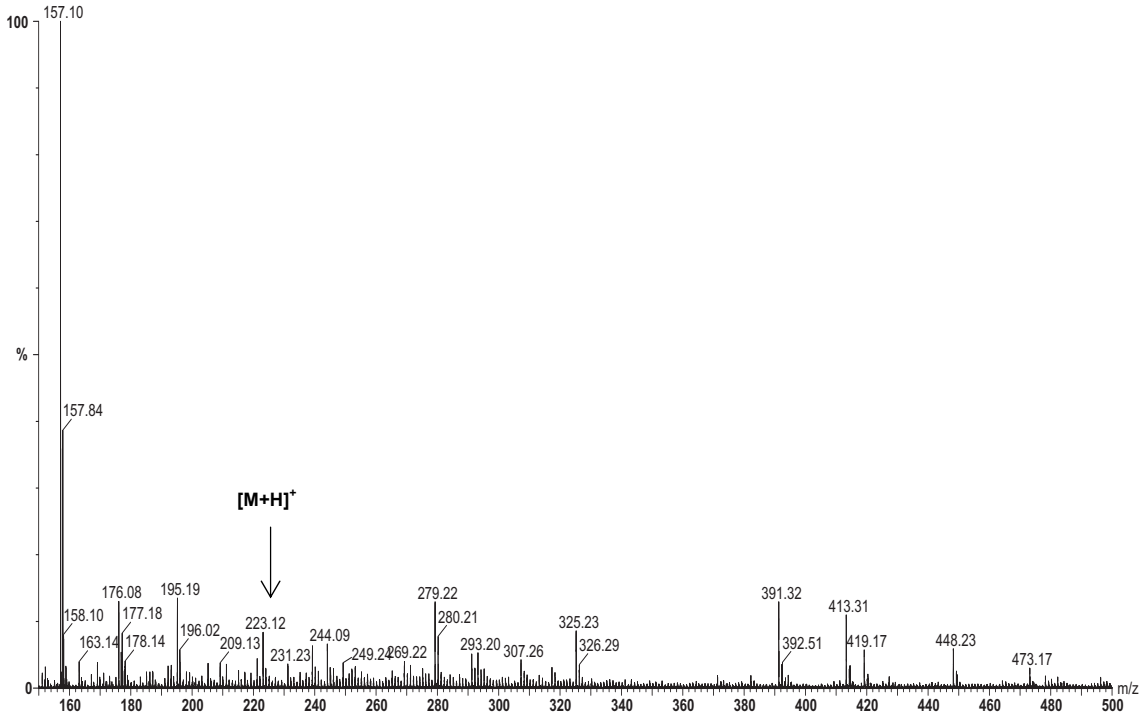
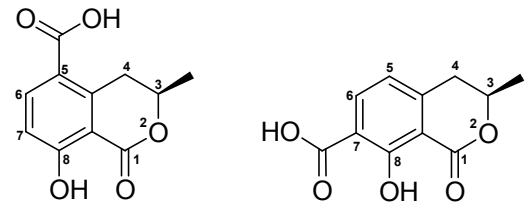


Figure 7.31: ESIMS spectrum of compound NAM 7-16.



(7.16) **(7.19)**

The ^{1}H NMR data of compound NAM 7-16 were compared to those reported for 5-carboxymellein (**7.16**) (Anderson and Edwards, 1983) and ochratoxin β (**7.19**) (Kraus, 1981) and these are presented in Table 7.3. Based on the comparison, the data of compound NAM 7-16 were consistent with those for 5-carboxymellein (**7.16**), and was thus identified as 5-carboxymellein (**7.16**).

Table 7.3: Comparison of ^{1}H NMR data of compound NAM 7-16 and of the reported compounds **7.16** and **7.19**.

Position	**δ^{1}H, ppm multiplicity (J_{HH} Hz)**		
	NAM 7-16[a]	**7.19 [b]**	**7.16[c]**
1			
2			
3	4.71, m	4.63 – 4.76, m	4.76, m
4	3.03, dd (11.8, 17.8)	2.8 – 3.2, m	3.02, dd (12.0, 17.5)
	3.90, dd (3.2, 17.8)		3.80, dd (3.0, 17.5)
5	6.93, d (9.0)	6.85, d (8.0)	6.96, d (9.0)
6	8.15, d (9.0)	7.95, d (8.0)	8.07, d (9.0)
7			
8			
3'	1.51, d, 3H, (6.2)	1.40, d (6.65)	1.45, d (5.5)

[a] These data were recorded at 500 MHz in CD_3OD; [b] These data were recorded in DMSO-d_6 (Kraus, 1981); [c] These data were recorded at 500 MHz in DMSO-d_6 (Anderson and Edwards, 1983).

7.4 Discussion

The dereplication approach using the LC-MS-UV works efficiently if the compounds have characteristic UV spectra, simple MS and not too many hits in the AntiMarin database search. A conclusive molecular weight cannot always be obtained from a single positive ion ESIMS experiment. Too often it is not possible to determine if the ion is MH^+ or MNa^+ or MNH_4^+ or $MHCH_3CN^+$ etc. (e.g: Nielsen and Smedsgaard, 2003) which can lead to confusion over the actual molecular weight. A good example of simple application of LC-MS-UV technique in combination with the AntiMarin database was presented by the identification of chromomycin A2 (**7.1**), chromomycin A3 (**7.2**) and chromomycin 02-3D (**7.3**) from extract F5956 (see **Section 7.2.1**). The AntiMarin database search (<50 hits) greatly aided the identification process. In the case of fungal metabolite, particularly from *Penicillium* spp., the incorporation of the published database for fungal metabolites (Nielsen and Smedsgaard, 2003) was useful in the process even if the AntiMarin database search resulted in too many hits (e.g: >150 hits). The significant peaks could be dereplicated by comparing their MS and UV profiles with the published database. UV spectral data was shown to be a valuable tool for identification of metabolites with similar MS data. This was seen in compounds NAM 7-7 (**Section 7.2.3**) and NAM 7-10 (**Section 7.2.4**) where both compounds showed similar presence of a dimeric cluster ion $[2M+H]^+$ that allowed assignment of their $[M+H]^+$ ions as 281. These compounds were characterized as cyclopeptin (**7.7**) (NAM 7-7) and brefeldin A (**7.10**) (NAM 7-10) based on the differences in their distinguished UV spectra.

The CapNMR technique together with the AntiMarin database enables the dereplication of metabolites without having to put effort for total purification of metabolites. Partially purified compounds in less than 10 µg could be effectively dereplicated using the power of the AntiMarin database. AntiMarin includes NMR-based functional group and many types of methyl groups and aromatic spin systems could be usually recognized in the ^{1}H NMR of partially pure compounds. Partially pure compounds NAM 7-14 (2 µg) (**Section 7.3.1**) and NAM 7-16 (3 µg) (**Section 7.3.2.2**) were successfully dereplicated as thiazostatin B (**7.14**) and 5-carboxymellein (**7.16**), respectively, using the CapNMR technique. In addition to that, a reasonable pure compound NAM 7-17 (2 µg) (**Section 7.3.2.1**) was identified as methyl 1*H*-indole-3-carboxylate (**7.17**). The incorporation of ^{1}H NMR data in the search profile of the database for compound NAM 7-14 reduced the possible hits to 19 from 218 hits of the original search by using only MS data. The indication of the presence of an N-methyl or aromatic methyl in compound NAM 7-14 further reduced the possible 19 hits to two. Similarly, the search profile for compound NAM 7-16 was reduced from 208 to 14 hits with known compounds. By considering the coupling constant pattern of the aromatic protons in compound NAM 7-16, the search was narrowed even further, so that the total number of possible structures was reduced from 14 to two.

Rapid dereplication of fungal metabolites was greatly supported by the in-house HPLC-UV/R_t library database. In this technique, a match with both the UV data and R_t was necessary for dereplication of a compound. In cases where related compounds with identical UV chromophores but differed in the R_t, further MS or NMR analysis needs to be carried out for confirmation. An example is shown by compound NAM 7-17 that displayed a UV match with the known indole-3-carboxylic acid (**7.15**) in the in-house library, but the R_t differed significantly. The ^{1}H NMR and MS data of compound NAM 7-17 were found to be in agreement with another type of indole compound, methyl 1*H*-indole-3-carboxylate (**7.17**).

Chapter 8

Conclusion

A series of experiments was conducted to investigate biological and chemical properties of marine-derived actinomycetes and fungi from various marine-derived invertebrates in New Zealand and Malaysian waters.

Actinomycetes and fungi were successfully isolated from both New Zealand and Malaysian marine invertebrates. All isolates grew on both sea water and non-sea water media, thus all of them can be classified as facultatively marine. No obligate marine microorganisms were isolated.

Some 55% of extracts showed some bioactivity. Of these, 93% showed cytotoxicity against the P388 cell lines and the remainder showed variable cytotoxic and antibacterial and/or antifungal activity.

The actinomycetes isolated differed from the fungi in that a clear preference was evident in the production of cytotoxic metabolites for solid-state fermentation as opposed to liquid fermentation. Fungi were very variable in this respect.

Three New Zealand actinomycetes, four New Zealand fungi and a Malaysian fungus whose initial screening showed good cytotoxicity (IC_{50}<12,500 ng/mL) were selected for bioassay guided chemical characterization. A Malaysian fungus that showed IC_{50}12,881 ng/mL was also investigated for chemical characterization due to interesting chemical properties.

Structural elucidation of metabolites was greatly aided by the in-house dereplication techniques using HPLC-UV/R_t library and AntiMarin database. A significant advantage was gained by the use of the CapNMR which enabled NMR characterization of very small quantities of metabolites (<20 μg).

A new metabolite, *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate (**5.15**) and four known metabolites (bohemamine (**5.1**), bohemamine B (**5.2**), montagnetol (**5.16**) and erythrin (**5.18**)) were isolated and identified from *Streptomyces* sp. (LA3L2) using the CapNMR technique. The structure of *S*-methyl 2,4-dihydroxy-6-isopropyl-3,5-dimethylbenzothioate (**5.15**) was fully characterized by ^{1}H, HSQC, HMBC and NOE experiments. This new compound is only the third compound reported to contain the *S*-methyl benzothioate group. *Streptomyces* sp. (LA3L2) is the first reported actinomycete to produce montagnetol (**5.16**) and erythrin (**5.18**). The known metabolite bohemamine (**5.1**) was produced significantly at a temperature 20 – 28ºC and 4% salinity.

The known compounds thiazostatin B (**7.14**) and three chromomycins (chromomycin A2 (**7.1**), A3 (**7.2**) and 02-3D (**7.3**)) were isolated from *Streptomyces* sp. (LA5L4) and *Streptomyces* sp. (LA3L1), respectively.

Penicillium sp. (LY1L5) yielded two known metabolites, cycloaspeptide A (**7.4**) and α-cyclopiazonic acid (**7.5**). α-Cyclopiazonic acid (**7.5**) and three other known metabolites (roquefortine A (**7.6**), cyclopeptin (**7.7**) and viridicatin (**7.8**)) were dereplicated from *Penicillium* sp. (KK3T23). *Penicillium* sp. (KK3T8) produced brefeldin A (**7.10**) while mycophenolic acid (**7.12**) and brevianamide A (**7.11**) were produced by *Penicillium* sp. (KK4T14b).

The effect of salinity on growth and cytotoxicity was investigated for the two *Penicillium* isolates producing the cytotoxic metabolite, α-cyclopiazonic acid (**7.5**). Results indicated that saline conditions were not required for growth but metabolite production differed between the two isolates with respect to salinity. Isolate LY1L5 required saline conditions for α-cyclopiazonic production whereas isolate KK3T23 produced the metabolite under non-saline conditions and in concentrations of sea salt up to 6%.

Three known metabolites, indole-3-carboxylic acid (**7.15**), indole-3-carboxylate (**7.17**) and 5-carboxymellein (**7.16**) were identified from *Paecilomyces* sp. (PR5L9). A unique cyclic depsipeptide structure, *epi*-angolide (NAM 6-1) was characterized from another *Paecilomyces* sp. (PR10T2) by ^{1}H, COSY, HSQC and HMBC experiments.

Rapid identification of known fungal metabolites enabled the in-house HPLC-UV/R_t library to be enhanced by eight metabolites. An HPLC-UV/R_t library for actinomycete metabolites was successfully established with the insertion of eight known metabolites.

References

Abraham, E. P. and Loder, P. B. 1972. In. Flynn, E. H. (ed). *Cephalosporins and Penicillins: Chemistry and Biology.* Academic Press. New York.

Abrell, L. M., Cheng, X. and Crews, P. 1994. *Tetrahedron Letters.* 35:9159-9160.

Abrell, L. M., Borgeson, B. and Crews, P. 1996. *Tetrahedron Letters.* 37:8983-8984.

Adinarayana, G., Venkateshan, M. R., Bapiraju, V. V. S. N. K., Sujatha, P., Premkumar, J., Ellaiah, P. and Zeeck, A. 2006. *Russian Journal of Bioorganic Chemistry.* 32:328-334.

Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffmann, C., Rothcock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, J. 1980. *Proceedings of the National Academy of Sciences of the United States of America.* 77:3957-3961.

Amagata, T., Doi, M., Ohta, T., Minoura, K. and Numata, A. 1998. *Journal of the Chemical Society, Perkin Transactions 1.* 21:3585-3600.

Amagata, T., Amagata, A., Tenney, K., Valeriote, F.A., Lobkovsky, E., Clardy, J. and Crews, P. 2003. *Organic Letters.* 23:4393-4396.

Amagata, T., Minoura, K. and Numata, A. 2006a. *Journal of Natural Products.* 69:1384-1388.

Amagata, T., Morinaka, B.I., Amagata, A., Tenney, K., Valeriote, F.A., Lobkovsky, E., Clardy, J. and Crews, P. 2006b. *Journal of Natural Products.* 69:1560-1565.

Anderson, J. R. and Edwards, R. L. 1983. *Journal of the Chemical Society, Perkin Transactions 1.* 2185-2192.

Asolkar, R. N., Jensen, P. R., Kauffman, C. A. and Fenical, W. 2006. *Journal of Natural Products.* 69:1756-1759.

Atz, J. W. 1952. *Animal Kingdom*. 55:175–177.

Barcina, I., Iriberri, J. and Egea, L. 1987. *Systematic and Applied Microbiology*. 10:85–91.

Barghoorn, E. S. 1944. *Farlowia.* 1:434-467.

Barton, S. D. and Nakanishi, K. 1999. *Comprehensive Natural Products Chemistry.* Elsevier, New York.

Belofsky, G. N., Jensen, P. R. and Fenical, W. 1999. *Tetrahedron Letters*. 40:2913-2916.

Bergmann, W. and Feeney, R. J. 1951. *Journal of Organic Chemistry.* 16:981-987.

Bewley, C. A., Holland, N. D. and Faulkner, D. J. 1996. *Experientia*. 52:716-722.

Bewley, C. A. and Faulkner, D. J. 1998. *Angewandte Chemie International Edition*. 37:2163-2178.

Bin Hamat, A. L., Bin Din, L., Bin Samsudin, M, W. and Elix, J. A. 1992. *Australian Journal of Chemistry.* 46:153-156.

Bister, B., Bischoff, D., Strobele, M., Riedlinger, J., Reicke, A., Wolter, F., Bull, A. T., Zahner, H., Fiedler, H. P., Sussmuth, R. D. 2004. *Angewandte Chemie International Edition.* 43:2574-2576.

Bloomberg, A. and Adler, A. 1992. *Advances in Microbial Physiology*. 33:144-212.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T. and Prinsep, M. R. 2003. *Natural Product Reports*. 20:1-48.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T. and Prinsep, M. R. 2004. *Natural Product Reports*. 21:1-49.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T. and Prinsep, M. R. 2005. *Natural Product Reports*. 22:15-61.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T. and Prinsep, M. R. 2006. *Natural Product Reports*. 23:26-78.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T. and Prinsep, M. R. 2007. *Natural Product Reports*. 24:31-86.

Bode, H. B., Bethe, B., Hofs, R. and Zeeck, A. 2002. *European Journal of Chemical Biology.* 3:619-627.

Bonnard, I., Manzanares, I. and Rinehart, K. L. 2003. *Journal of Natural Products.* 66:1466-1470.

Boot, C.M., Tenney, K., Valeriote, F. A. and Crews, P. 2006. *Journal of Natural Products.* 69:83-92.

Borris, R. P. and Gould, S. J. 1999. *Molecular Diversity, Biological Diversity and the Search for New Drugs.*
URL: http://www.iupac.org/symposia/proceedings/phuket97/borris.html

Brauers, G., Ebel, R., Edrada R. A., Wray, V., Berg, A., Grafe, U. and Proksch, P. 2001. *Journal of Natural Products.* 64:651-652.

Bringmann, G., Lang, G., Muhlbacher, J., Schaumann, K., Steffens, S., Rytik, P.G., Hentschel, U., Morschhauser, J. and Muller, W.E.G. 2003. *Progress in Molecular and Subcellular Biology*. 37:231-253.

Bringmann, G., Lang, G., Steffens, S. and Schaumann, K. 2004. *Journal of Natural Products.* 67:311-315.

Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L. and Korn, E. D. 1994. *Journal of Biological Chemistry*. 269:14869-14871.

Buchanan, G. O., Williams, P. G., Feling, R. H., Kauffman, C. A., Jensen, P. R., Fenical, W. 2005. *Organic Letters*. 7:2731-2734.

Bugni, T. S., Abbanat, D., Bernan, V. S., Maiese, W. M., Greenstein, M., Van Wagoner, R. M. and Ireland, C. M. 2000. *Journal of Organic Chemistry*. 65:7195-7200.

Bugni, T. S. and Ireland, C. M. 2004. *Natural Product Reports*. 21:143-163.

Bugni, T. S., Woolery, M., Kauffman, C. A., Jensen, P. R. and Fenical, W. 2006. *Journal of Natural Products.* 69:1626-1628.

Bull, A. T., Ward, A. C., and Goodfellow, M. 2000. *Microbiology and Molecular Biology Reviews.* 64:573–606.

Burja, A. M., Banaigs, B., Abou-Mansour, E., Burgess, J. G. and Wright, P. C. 2001. *Tetrahedron*. 57:9347

Cabrera, G. M., Butler, M., Rodriguez, M. A., Godeas, A. H., Haddad, R. and Eberlin, M. N., 2006. *Journal of Natural Products*. 69:1806-1808.

Capon, R. J., Skene, C. Stewart, M., Ford, J., O'Hair, R. A. J., Williams, L., Lacey, E., Gill, J. H., Heiland, K. and Friedel, T. 2003. *Organic and Biomolecular Chemistry*. 1:1856-1862.

Chan, W. R., Tinto, W. F., Manchand, P. S. and Todaro, L. J. 1987. *Journal of Organic Chemistry.* 52:3091-3093.

Charan, R.D., Schlingmann, G., Janso, J., Bernan, V., Feng, X., Carter, G.T. 2004. *Journal of Natural Products.* 67:1431-1433.

Chauhan, D., Catley, L., Li, G., Podar, K., Hideshima, T., Velankar, M., Mitsiades, C., Mitsiades, N., Yasui, H., Letai, A. 2005. *Cancer Cell.* 8:407-419.

Cheng, X., Varoglu, M., Abrell, L., Crews, P., Lobkovsky, E. and Clardy, J. 1994. *Journal of Organic Chemistry.* 59:6344-6348.

Cheng, Y., Schneider, B., Riese, U., Schubert, B., Li, Z. and Hamburger, M. 2006. *Journal of Natural Products*. 69:436-438.

Cho, J. Y., Kwon, H. C., Williams, P. G., Jensen, P. R. and Fenical, W. 2006. *Organic Letters.* 8:2471-2474.

Christian, O. E., Compton, J., Christian, K. R., Mooberry, S. L., Valeriote, F. A. and Crews, P. 2005. *Journal of Natural Products*. 68:1592-1597.

Colquhoun, J. A., Mexson, J., Goodfellow, M., Ward, A. C., Horikoshi, K. and Bull, A. T. 1998. *Antonie van Leeuwenhoek*. 74:27–40.

Crews, P., Manes, L.V. and Boehler, M. 1986. *Tetrahedron Letters.* 27:2797.

Cruz, L. J., Martinez, I. M., Perez Baz, J., Trujillo, M., Rodriquez-Miaz, R. A., Oliveira, E., Giralt, E., Albericio, F. and Canedo, L. M. 2006. *Journal of Organic Chemistry*. 71:3335-3338.

Cueto, M., Jensen, P. R. and Fenical, W. 2000. *Phytochemistry*. 55:223-226.

Culberson, C. F. 1969. *Chemical and Botanical Guide to Lichen Products.* The University of North Carolina Press. Chapel Hill. USA. pp. 112-117.

Culvenor, C. C. J. 1980. In. Smith, R. L. and Bababunmi, E. A. (ed.). *Toxicology in the Tropics.* Taylor and Francis Limited. London. pp. 124-141.

Dalsgaard, P. W., Larsen, T. O., Frydenvang, K. and Christophersen, C. 2004. *Journal of Natural Products*. 67:878-881.

Dalsgaard, P. W., Larsen, T. O. and Christophersen, C. 2005. *Journal of Antibiotics*. 58:141–144.

Demain, A. L. 1968. *Lloydia*. 31:395-418.

Doyle, T. W., Nettleton, D. E. Jr., Balitz, D. M. Moseley, J. E., Grulich, R. E., McCable, T. and Clardy, J. 1980. *Journal of Organic Chemistry.* 45:1324-1326.

Dreyfuss, M., Harri, E., Hotmann, H., Kobel, H., Pache, W. and Tscherter, H. 1976. *Journal of Applied Microbiology*. 3:125-133.

Ebel, R. 2006. In. Proksch, P. and Muller, W. E. G. (eds.). *Frontiers in Marine Biotechnology.* Horizon Bioscience. pp. 73-143.

Edrada, R. A., Wray, V., Berg, A., Grafe, U., Sudarsono, Brauers, G. and Proksch, P. 2000. *Zeitschrift fur Naturforschung C: Journal of Biosciences.* 55:218-221.

Edrada, R. A., Heubes, M., Brauers, G., Wray, V., Berg, A., Grafe, U., Wohlfarth, M., Muhlbacher, J., Schaumann, K., Sudarsono, Bringmann, G. and Proksch, P. 2002. *Journal of Natural Products*. 65:1598-1604.

Ellis, M. B., Riches, P. and Russel, D. W. 1966. *Biochemical Journal.* 98:8P-9P.

Elix, J. A., Kalb, K. and Wardlaw, J. H. 2003. *Australian Journal of Chemistry.* 56:315-317.

Espinosa, R. M. D. and Webb, C. 2003. *World Journal of Microbiology and Biotechnology.* 19:329-336.

Farrel, J. and Rose, A. 1967. *Annual Review of Microbiology*. 21:101-120.

Faulkner, D. J. 1994. *Natural Product Reports.* 11:355-394.

Faulkner, D. J. 1999. *Natural Product Reports.* 16:155-198.

Faulkner, D. J. 2000a. *Natural Product Reports.* 17:1-6.

Faulkner, D. J. 2000b. *Natural Product Reports.* 17:7-55.

Faulkner, D. J. 2001. *Natural Product Reports.* 18:1-49.

Faulkner, D. J. 2002. *Natural Product Reports.* 19:1-48.

Feling, R. H., Buchanan, G. O., Mincer, T. J., Kauffman, C. A., Jensen, P. R., Fenical, W. 2003. *Angewandte Chemie International Edition England.* 42:355-357.

Fenical, W. 1993. *Chemical Reviews.* 93:1673–1683.

Fenical, W. 1997. *Trends in Biotechnology*. 15:339-341.

Fernandez, R., Omar, S., Feliz, M., Quinoa, E. and Riguera, R. 1992. *Tetrahedron Letters.* 33:6017-6020.

Fiedler, H. P., Bruntner, C., Bull, A. T., Ward, A. C, Goodfellow, M., Potterat, O., Puder, C. and Mihm, G. 2005. *Antonie van Leeuwenhoek*. 87:37-42.

Filtenbort, O., Frisvad, J. C. and Svendsen, J. A. 1983. *Appllied and Environmental Microbiology*. 45:581–585.

Ford, P. W., Gadepalli, M. and Davidson, B. S. 1998. *Journal of Natural Products*. 61:1232-1236.

Frendenhagen, A., Hug, P., Sauter, H. and Peter, H. H. 1995. *Journal of Antibiotics*. 48:199-204.

Frisvad, J. and Thrane, U. 1987. *Journal of Chromatography*. 404:195.

Frisvad, J. C., Filtenborg, O. and Thrane, U. 1989. *Archives of Environmental Contamination and Toxicology.* 18:331–335.

Frisvad, J. C., Smedsgaard, J., Larsen, T. O. and Samson, R. A. 2004. *Studies in Mycology.* 49:201–242.

Frisvad, J. C., Larsen, T. O., Dalsgaard, P. W., Seifert, K. A., Seize, G. L., Lyhne, E. K., Jarvis, B. B., Fettinger, J. C. and Overy, D. P. 2006. *International Journal of Systematic and Evolutionary Microbiology.* 56:1427-1437.

Fukushima, K., Arai, T., Mori, Y., Tsuboi, M. and Suzuki, M. 1983a. *Journal of Antibiotics*. 36:1606-1612.

Fukushima, K., Arai, T., Mori, Y., Tsuboi, M. and Suzuki, M. 1983b. *Journal of Antibiotics*. 36:1613-1630.

Furuya, K. 1986. In. Yamasato, H., Udagawa, S., Kodama, T. and Morichi, T. (eds.). *Marine fungi – Isolation Methods of Microorganisms.* R & D Planning. Tokyo. pp. 101.

Gonzalez, A. G., Bermejo, B. J., Rodriguez, P. E. M. and Hernandez, P. C. E. 1992. *Planta Medica.* 58:214-218.

Gonzalez, J. B., Fernandez, F. J. and Tomasini, A. 2003. *Indian Journal of Biotechnology.* 2:322-333.

Gronquist, M., Meinwald, J., Eisner, T. and Schroeder, F.C. 2005. *Journal of the American Chemical Society.*127:10810-10811.

Haefner, B. 2003. *Drug Discovery Today*. 8:536–544.

Harris, R. F. 1981. In. Parr, J. F., Gardner, W. R. and Elliot, L. F. (eds.). *Water Potential Relations in Soil Microbiology.* Soil Science Society of America. Madison. Wisconsin. p. 23-95.

Higgs, R. E., Zahn, J. A., Gygi, J. D. and Hilton, M. D. 2001. *Applied and Environmental Microbiology.* 67:371–376.

Hiort, J., Maksimenka, K., Reichert, M., Perovic-Ottstadt, Lin, W. H., Wray, V., Steube, K., Schaumann, K., Weber, H., Proksch, P., Ebel, R., Muller, W. E. G. and Bringmann, G. 2004. *Journal of Natural Products.* 67:1532-1543.

Holler, U., Konig, G. M. and Wright, A. D. 1999. *Journal of Natural Products.* 62:114-118.

Hu, J. F., Wunderlich, D., Thiericke, R., Dahse, H. M., Grabley, S., Feng, X. Z., Sattler, I. 2003. *Journal of Antibiotics*. 56:747-754.

Hu, J. F., Garo, E., Yoo, H. D., Cremin, P. A., Zeng, L., Goering, M. G., Johnson, M. O. and Eldridge, G. R. 2005. *Phytochemical Analysis*. 16:127–133.

Huang, Y. F., Tian, L., Fu, H. W., Hua, H. M. and Pei, Y. H. 2006. *Natural Products Research*. 20:1207-1210.

Hughes, G.C. 1975. *Oceanographic and Marine Biology Annual Review.* 13:69-180.

Huneck, S. and Follmann, G. 1968a. *Bryologist.* 71:266-267.

Huneck, S. and Follmann, G. 1968b. *Pharmazie*. 23:156-157.

Imada, C. and Okami, Y. 1995. *Journal of Marine Biotechnology*. 2:109–113.

Isaka, M., Kittakoop, P. and Thebtaranonth, Y. 2003. In. White, J. F., Jr., Bacon, C. W., Hywel-Jones, N. L. and Spatafora, J. W. (ed.). *Clavicipitalean Fungi: Evolutionary Biology, Chemistry, Biocontrol and Cultural Impacts.* Marcel Dekker Incorporation. New York. p. 355-398.

Isaka, M., Palasarn, S., Lapanun, S. and Sriklung, K. 2007. *Journal of Natural Products*. 70:675-678.

Itoh, T., Kinoshita, M., Aoki, S. and Kobayashi, M. 2003. *Journal of Natural Products*. 66:1373-1377.

Jadulco, R., Proksch, P., Wray, V., Sudarsono, Berg, A. and Graefe, U. 2001. *Journal of Natural Products*. 64:527-530.

Jadulco, R., Brauers, G., Edrada, R. A., Ebel, R., Wray, V., Sudarsono and Proksch, P. 2002. *Journal of Natural Products*. 65:730-733.

Jadulco, R., Edrada, R. A., Ebel, R., Berg, A., Schaumann, K., Wray, V., Staube, K. and Proksch, P. 2004. *Journal of Natural Products*. 67:78-81.

Jansen, R., Kunze, B., Reichenbach, H. and Hofle, G. 1996. *Liebigs Annalen.* 2:285-290.

Janso, J. E., Bernan, V. S., Greenstein, M., Bugni, T. S. and Ireland, C. M. 2005. *Mycologia.* 97:444-453.

Jenkins, K. M., Renner, M. K., Jensen, P. R. and Fenical, W. 1998. *Tetrahedron Letters*. 39:2463-2466.

Jennings, D. H. 1983. *Biological Review.* 58:423-459.

Jensen, P. R., Dwight, R. and Fenical, W. 1991. *Applied and Environmental Microbiology.* 57:1102–1108.

Jensen, P. R., Mincer, T. J, Williams, P. G and Fenical, W. 2005a. *Antonie van Leeuwenhoek*. 87:43-48.

Jensen, P. R., Gontang, E., Mafnas, C., Mincer, T. J. and Fenical, W. 2005b. *Environmental Microbiology.* 7:1039-1048.

Jones, E. B. G. and Jennings, D. H. 1964. *Transactions of British Mycological Society*. 47:619-625.

Kathirgamanathar, S., Williams, D. E., Andersen, R. J., Bombuwela, K., De Silva, D., Karunaratne, V. 2005. *Natural Products Research.* 19:695-701.

Kawano, T., Hidaka, T., Furihata, K., Mochizuki, J., Nakayama, H., Hayakawa, Y. and Seto, H. 1990. *Journal of Antibiotics*. 43:110-113.

Kelecom A. 1999. *Anais da Academia Brasileira de Ciencias*. 71:249-263.

Kelecom, A. 2002. *Anais da Academia Brasileira de Ciencias*. 74:151–170.

Kikuchi, H., Miyagawa, Y., Sahashi, Y., Inatomi, S., Haganuma, A., Nakahata, N. and Oshima, Y. 2004a. *Tetrahedron Letters*. 45:6225-6228.

Kikuchi, H., Miyagawa, Y., Nakamura, K., Sahashi, Y., Inamori, S. and Oshima, Y. 2004b. *Organic Letters*. 6:4531-4533.

Kikuchi, H., Miyagawa, Y., Sahashi, Y., Inatomi, S., Haganuma, A., Nakahata, N. and Oshima, Y. 2004c. *Journal of Organic Chemistry*. 69:352-356.

Kitagawa, I., Nishino, T., Kobayashi, M. and Kyogoku, Y. 1981. *Chemical and Pharmaceutical Bulletin.* 29:1951–1956.

Klaric, M. S. and Pepeljnjak, S. 2005. *Arhiv Za Higijenu Rada I Toksikologiyu.* 56:343-350.

Kobayashi, R., Samejima, Y., Nakajima, S., Kawai, K. I. and Udagawa, S. I. 1987. *Chemical and Pharmaceutical Bulletin*. 35:1347-1352.

Kobayashi, M., Kuroso, M., Ohyabu, N., Wang, W., Fujii, S. and Kitagawa, I. 1994a. *Chemical and Pharmaceutical Bulletin*. 42:2196-2198.

Kobayashi, M., Aoki, S., Ohyabu, N., Kuroso, M., Wang, W. and Kitagawa, I. 1994b. *Tetrahedron Letters*. 35:7969-7972.

Kohlmeyer, J. 1974. *Veroeff. Inst. Meeresforsch. Bremerhaven. Suppl.* 5:263-286.

Kohlmeyer, J. and Kohlmeyer, E. 1979. *Marine Mycology: The Higher Fungi.* Academic Press. New York.

Kokare, C. R., Mahadik, K. R., Kadam, S. S. and Chopade, B. A. 2004. *Current Science*. 86:593-597.

Kralj, A., Kehraus, S., Krick, A., van Echten-Deckert, G. and Koenig, G. M. 2007. *Planta Medica*. 73:366-371.

Kraus, G. A. 1981. *Journal of Organic Chemistry.* 46:201-202.

Kupka, J., Anke, T., Steglich, W. and Zechlin, L. 1981. *Journal of Antibiotics*. 34:298-304.

Laatsch, H. 2006. In. Proksch, P. and Muller, W. E. G. (eds.). *Frontiers in Marine Biotechnology.* Horizon Bioscience. p. 225-288.

Lam, K. S. 2006. *Current Opinion in Microbiology.* 9:245–251.

Landreau, A., Pouchus, Y. F., Sallenave-Namont, C., Biard, J. F., Boumard, M. C., Pont, T. R., Mondeguer, F., Goulard, C. and Verbist, J. F. 2002. *Journal of Microbiological Methods*. 48:181–194.

Lang, G., Blunt, J. W., Cummings, N. J., Cole, A. L. J. and Munro, M. H. G. 2005. *Journal of Natural Products*. 68:810-811.

Lang, G., Mitova, M. I., Ellis, G., van der Sar, S., Phipps, R. K., Blunt, J. W., Cummings, N. J., Cole, A. L. J. and Munro, M. H. G. 2006. *Journal of Natural Products.* 69:621-624.

Lang, G., Wiese, J., Schmaljohann, R. Adn Imhoff, J. F. 2007. *Tetrahedron.* 63:11844-11849.

Lee, H. K., Lee, D. S., Lim, J., Kim, J. S., Im, K. S. and Jung, J. H. 1998. *Archives of Pharmacal Research*. 21:729-733.

Lee, H. S., Shin, H. J., Jang, K. H., Kim, T. S., Oh, K. B. and Shin, J. 2005. *Journal of Natural Products.* 68:623-625.

Li, F., Maskey, R. P., Qin, S., Sattler, I., Fiebig, H. H., Maier, A., Zeeck, A. and Laatsch, H. 2005. *Journal of Natural Products.* 68:349-353.

Lin, Y., Shao, Z., Jiang, G., Zhou, S., Cai, J., Vrijmoed, L. L. P. and Jones, E. B. G. 2000. *Tetrahedron.* 56:9607-9609.

Lin, W., Brauers, G., Ebel, R., Wray, V., Berg, A., Sudarsono and Proksch, P. 2003. *Journal of Natural Products.* 66:57-61.

Lopez, J. M. S., Insua, M. M., Baz, J. P., Puentes, J. L. F. and Hernandez, L. M. C. 2003. *Journal of Natural Products.* 66:863-864.

Luesch, H., Moore, R. E., Paul, V. J., Mooberry, S. L. and Corbett, T. H. 2001. *Journal of Natural Products.* 64:907-910.

Macdonald, C. G. and Shannon, J. S. 1964. *Tetrahedron Letters*. (41-42):3113-3118.

Macherla, V. R., Liu, J., Bellows, C., Teisan, S., Nicholson, B., Lam, K. S. and Potts, B. C. M. 2005. *Journal of Natural Products.* 68:780-783.

Magarvey, N. A., Keller, J.M., Bernan, V., Dworkin, M. and Sherman, D. H. 2004. *Applied and Environmental Microbiology*. 70:7520-7529.

Maldonado, L. A., Fenical, W., Jensen, P. R., Kauffman, C. A., Mincer, T. J., Ward, A. C., Bull, A. T. and Goodfellow, M. 2005. *International Journal of Systematic and Evolutionary Microbiology.* 55:1759-1766.

Manam, R. R., Teisan, S., White, D. J., Nicholson, B., Grodberg, J., Neuteboom, S. T. C., Lam, K. S., Mosca, D. A., Lloyd, G. K. and Potts, B. C. M. 2005. *Journal of Natural Products.* 68:240-243.

Masuma, R., Yamaguchi, Y., Noumi, M., Omura, S. and Namikoshi, M. 2001. *Mycoscience*. 42:455-459.

Mikami, Y., Yazawa, K., Fukushima, K., Arai, T., Udagawa, S. and Samson, R. A. 1989. *Mycopathologia*. 108:195-199.

Miller, J. D. and Savard, M. E. 1989. *Proceedings of the Nova Scotia Institute of Science*. 39:51-58.

Miller, E. D., Kauffman, C. A., Jensen, P. R. and Fenical, W. 2007. *Journal of Organic Chemistry.* 72:323-330.

Mincer, T. J., Jensen, P. R., Christopher, A. K. and Fenical, W. 2002. *Applied and Environmental Microbiology*. 68:5005–5011.

Mincer, T. J., Fenical, W. and Jensen, P. R. 2005. *Applied and Environmental Microbiology*. 71:7019-7028.

Mitchell, S. S., Nicholson, B., Teisan, S., Lam, K. S. and Potts, B. C. M. 2004. *Journal of Natural Products.* 67:1400-1402.

Miyamoto, M., Kawashima, K., Kawamatsu, Y., Shinohara, M., Tanaka, K., Tatsuoka, S. and Nakanishi, K. 1967. *Tetrahedron.* 23:421-437.

Moore, B. S. 1999. *Natural Product Reports*. 16:653-674.

Munoz, G. M. D. 2006. *MSc Thesis.* University of Puerto Rico. Mayaguez Campus.

Namikoshi, M., Kobayashi, H., Yoshimoto, T. and Meguro, S. 2000. *Chemistry Letters*. 4:308-309.

Namikoshi, M., Akano, K., Kobayashi, H., Koike, Y., Kitazawa, A., Rondonuwu, A. B. and Pratasik, S. B. 2002. *Journal of Tokyo University of Fisheries.* 88:15-20.

Nettleton, D. E., Jr., Balitz, D. M., Doyle, T. W.; Bradner, W. T., Johnson, D. L., O'Herron, F. A., Schreiber, R. H., Coon, A. B., Moseley, J. E., Myllymaki, R. W. 1980. *Journal of Natural Products.* 43:242-258.

Newman, D. J. and Cragg, G. M. 2004. *Journal of Natural Products.* 67:1216–1238.

Nielsen, K. F. and Smedsgaard, J. 2003. *Journal of Chromatography A.* 1002:111–136.

Nigrelli, R. F., Stempien, M. F., Ruggieri, G. D., Liguori, V. R. and Cecil, J. T. 1967. *Federation Proceedings*. 26:1197–1205.

Numata, A., Amagata, T., Minoura, K. and Ito, K. 1997. *Tetrahedron Letters.* 38:5675.

Oh, D. C., Jensen, P. R. and Fenical, W. 2006. *Tetrahedron Letters*. 47:8625-8628.

Okami, Y. 1974. *Journal of Antibiotics*. 27:240-247.

Okami, Y. and Hotta, K. 1988. In. Goodfellow, M., Williams, S. T. and Mordarski, M. (ed.). *Actinomycetes in Biotechnology.* Academic Press. New York. p. 33–67.

Okotore, R. O. and Russel, D. W. 1972. *Canadian Journal of Biochemistry.* 50:428-439.

Perry, N. B., Benn, M. H., Brennan, N. J., Burgess, E. J., Ellis, G., Galloway, D. J., Lorimer, S. D. and Tangney, R. S. 1999. *Lichenologist.* 31:627-636.

Pettit, G. R. 1997. *Fortschritte der Chemie Organischer Naturstoffe: Progress in the Chemistry of Organic Natural Products*.. 70:1-79.

Piel, J. 2004. *Natural Product Reports*. 21:519-538.

Pitt, J. I. 1979. *The Genus Penicillium and Its Teleomorphic States Eupenicillium and Talaromyces.* Academic Press. New York.

Pittayakhajonwut, P., Dramae, A., Madla, S., Lartpornmatulee, N., Boonyuen, N., Tanticharoen, M. 2006. *Journal of Natural Products*. 69:1361-1363.

Poch, G. K. and Gloer, J. B. 1991. *Journal of Natural Products*. 54:213-217.

Poumale, H. M. P., Ngadjui, B. T., Helmke, E. and Laatsch, H. 2006. *Zeitschrift fur Naturforschung B: Journal of Chemical Sciences.* 61:1450-1454.

Preobrazhenskaia, T. P., Kudrina, E. S., Sveshnikova, M. A. and Maximova, T. S. 1959. *Mikrobiologiya.* 28:623-627.

Proksch, P., Edrada, R. A. and Ebel, R. 2002. *Applied Microbiology and Biotechnology.* 59:125-134.

Proksch, P., Edrada, R. A. and Lin, W. H. 2006. In. Proksch, P. and Muller, W. E. G. (eds.). *Frontiers in Marine Biotechnology.* Horizon Bioscience. p. 1-19.

Pusecker, K., Laatsch, H., Helmke, E. and Weyland, H. 1997. *Journal of Antibiotics*. 50:479-483.

Rahbaek, L., Sperry, S., Piper, J. E., Crews, P. 1998. *Journal of Natural Products*. 61:1571-1573.

Rao, V. S. and Seshadri, T. R. 1941. *Proceedings – Indian Academy of Science, Section A.* 13A:199-202.

Rao, V. S. and Seshadri, T. R. 1942a. *Proceedings – Indian Academy of Science, Section A.* 15A:18-23.

Rao, V. S. and Seshadri, T. R. 1942b. *Proceedings – Indian Academy of Science, Section A.* 16A:23-28.

Rath, J. P., Kinast, S., Maier, M. E. 2005. *Organic Letters.* 7:3089-3092.

Rathnakala, R. R. and Chandrika, V. 1993. *Indian Journal of Marine Science*. 22:297-299.

Rehm, H. J. 1980. *Industrielle Mikrobiologie*. Springer-Verlag, Berlin.

Renner, M. K., Shen, Y. C., Cheng, X. C., Jensen, P. R., Frankmoelle, W., Kauffman, C. A., Fenical, W., Lobkovsky, E. and Clardy, J. 1999. *Journal of the American Chemical Society.* 121:11273-11276.

Rezanka, T. and Votruba, J. 1998. *Folia Microbiologica.* 43:47-50.

Riedlinger, J., Reicke, A., Zahner, H., Krismer, B., Bull, A. T., Maldonado, L. A., Ward, A. C., Goodfellow, M., Bister, B., Bischoff, D. 2004. *Journal of Antibiotics*. 57:271-279.

Rinehart, K. L. Jr., Gloer, J. B., Hughes, R. G. Jr., Renis, H. E., McGovren, J. P., Swynenberg, E. B., Stringfellow, D. A., Kuentzel, S. L. and Li, L. H. 1981. *Science*. 212:933-935.

Ritchie, D. 1957. *American Journal of Botany*. 44:870-874.

Roberts, M. F. 2005. *Saline Systems*. 1:5.
URL:http://www.salinesystems.org/content/1/1/5

Ruggieri, G. D. 1976. *Science*. 194:491-496.

Russel, D. W. 1965. *Journal of the Chemical Society.* Sept:4664-4668.

Russel, D. W. 1988. *Canadian Journal of Botany.* 66:1474-1475.

Sasaki, T., Igarashi, Y., Saito, N. and Furumai, T. 2002. *Journal of Antibiotics*. 55:249-255.

Schumacher, R. W., Talmage, S. C., Miller, S. A., Sarris, K. E., Davidson, B. S. and Goldberg, A. 2003. *Journal of Natural Products*. 66:1291-1293.

Selvin J., Joseph, S., Asha, K. R. T., Manjusha, W. A., Sangeetha, V. S., Jayaseema, D. M., Antony, M. C. and Vinitha, A. J. D. 2004. *FEMS Microbiology Ecology*. 50:117–122.

Sguros, P. L. and Simms, J. 1964. *Journal of Bacteriology*. 38:346-355.

Shao, N., Yao, G. and Chang, L. C. 2007. *Journal of Natural Products.* 70:869-871.

Shindo, K., Takenaka, A., Noguchi, T., Hayakawa, Y. and Seto, H. 1989. *Journal of Antibiotics.* 42:1526-1529.

Sitachitta, N., Gadepalli, M. and Davidson, B. S. 1996. *Tetrahedron*. 52:8073-8080.

Smedsgaard, J. and Frisvad, J. C. 1996. *Journal of Microbiological Methods.* 25:5-17.

Smith, C. J., Abbanat, D., Bernan, V. S., Maiese, W. M., Greenstein, M., Jompa, J., Tahir, A. and Ireland, C. M. 2000. *Journal of Natural Products.* 63:142-145.

Snider, B. B., Duvall, J. R., Sattlerb, I. and Huang, X. 2004. *Tetrahedron Letters.* 45:6725–6727.

Soman, A. G., Gloer, J. B. and Wicklow, D. T. 1999. *Journal of Natural Products.* 62:386-388.

Sonjak, S., Frisvad, J. C. and Cimerman, N. G. 2005. *FEMS Microbiology Ecology*. 53:51–60.

Stritzke, K., Schulz, S., Laatsch, H., Helmke, E. and Beil, W. 2004. *Journal of Natural Products.* 67:395-401.

Sultan, M. Z., Khatune, N. A., Sathi, Z. S., Bhuiyan, M. S. A., Sadik, M. G., Choudury, M. A., Gafur, M. A. and Rahman, M. A. A. 2002. *Biotechnology.* 1:100-106.

Suzuki, H., Tahara, M., Takahashi, M., Matsumura, F., Okabe, T., Shimazu, A., Hirata, A., Yamaki, H., Yamaguchi, H, Tanaka, N. and Nishimura, T. 1990. *Journal of Antibiotics*. 43:129-134.

Tahara, M., Okabe, T., Furihata, K., Tanaka, N., Yamaguchi, H., Nishimura, T. and Suzuki, H. 1990. *Journal of Antibiotics*. 43:135-137.

Tahara, M., Okabe, T., Furihata, K., Tanaka, N., Yamaguchi, H., Nishimura, T. and Suzuki, H. 1991. *Journal of Antibiotics*. 44:255.

Tan, J., Bednarek, P., Liu, J., Schneider, B., Svatos, A. and Hahlbrock, K. 2004. *Phytochemistry*. 65:691–699.

Van Eijk, G. W. and Roeijmans, H. J. 1984. *Experimental Mycology*. 8:266-268.

Vairappan, C. S., Daitoh, M., Suzuki, M., Abe, T. and Masuda, M. 2001. *Phytochemistry.* 58:291-297.

Vairappan, C. S. 2003. *Biomolecular Engineering*. 20:255-259.

Vairappan, C. S., Kawamoto, T., Miwa, H. and Suzuki, M. 2004. *Planta Medica.* 70:1087-1090.

Votruba, J. and Vanek, Z. 1989. In. Shapiro, S. (ed.) *Regulation of Secondary Metabolism in Actinomycetes.* CRC Press. p. 264 – 281.

Waksman, S. A. 1959. The Actinomycetes: *Nature, Occurrence and Activities.* Volume 1. Williams and Wilkins Co. Baltimore. p. 146-147.

Waksman, S. A. and Woodruff, H. B. 1940. *Proceedings of the Society for Experimental Biology and Medicine.* 45:609–614.

Walker, J. D. and Colwell, R. R. 1975. *Marine Biology.* 30:193–201.

Weinberg, E.D. 1974. Secondary Metabolism: Control by Temperature and Inorganic Phosphate. *Dev. Ind. Microbiology.* 15:70-81.

Weyland, H. 1969. *Nature* 223:858.

Wilkinson, C. R. 1992. In Reisser, W. (ed.). *Algae and Symbioses.* p. 112–151. Biopress. Bristol.

Williams, S. T., Lanning, S. and Wellington, E.M.H. 1984. In. Goodfellow, M., Mordarski, M. and Williams, S. T. (eds.). *The Biology of The Actinomycetes.* Academic Press. London. p. 481–528.

Xin, Z. H., Fang, Y., Du, L., Zhu, T., Duan, L., Chen, J., Gu, Q., and Zhu, W. 2007. *Journal of Natural Products*. 70:853-855.

Yamada, T., Minoura, K. and Numata, A. 2002. *Tetrahedron Letters.* 43:1721–1724.

Yang, L., Tan, R., Wang, Q., Huang, W. and Yin, Y. 2002. *Tetrahedron Letters.* 43:6545–6548.

Yang, R., Li, C., Lin, Y., Peng, G., She, Z. and Zhou, S. 2006. *Bioorganic and Medicinal Chemistry Letters.* 16:4205-4208.

Yin, S. W., Shi, Y. P., Li, X. M. and Wang, B. G. 2005. *Chinese Chemical Letters.* 16:1489-1491.

Yoshimura, Y., Koenuma, M., Matsumoto, K., Tori, K. and Terui, Y. 1988. *Journal of Antibiotics.* 41:53-67.

Yoshimura, I. Kinoshita, Y. Yamamoto, Y. Huneck, S. and Yamada, Y. 1994. *Phytochemical Analysis*. 5:197-205.

Yue, Q., Miller, C. J., White, J. F. and Richardson, M. D. 2000. *Journal of Agricultural and Food Chemistry.* 48:4687-4692.

Yutaka, E., Ushio, S. and Shoji, S. 1970. *Phytochemistry*. 9:2061-2063.

Zhang, W., Xue, S., Zhao, Q., Zhang, X., Li, J., Jin, M., Yu, X. and Yuan, Q. 2003a. *Biomolecular Engineering.* 20:413-419.

Zhang, Q., Schrader, K. K., El Sohly, H. N. and Takamatsu, S. 2003b. *Journal of Antibiotics*. 56:673-681.